RESPONSE SURFACE OPTIMIZATION OF *BACILLUS THURINGIENSIS ISRAELENSIS* FERMENTATION

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
THE MIDDLE EAST TECHNICAL UNIVERSITY

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

THE DEPARTMENT OF BIOLOGY

DECEMBER 2003

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ABSTRACT

RESPONSE SURFACE OPTIMIZATION OF *BACILLUS THURINGIENSIS ISRAELENSIS* FERMENTATION

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December 2003, 86 pages

The control of pest populations by using insect pathogens has been an attractive alternative to the application of chemical pesticides employed for the same purpose. As these chemicals not only damage the environment, but also trigger development of resistance by the pests and can harm other organisms together

with the target pest, biological control is preferable and *Bacillus thuringiensis (Bt)* subspecies have been the most widely used bioinsecticides in forestry, agriculture and mosquito/ black fly control.

The most important property of *Bt* subspecies is the synthesis of protoxins named as delta-endotoxins (crystal proteins). In this study, response surface optimization of *Bt* subsp. *israelensis* HD500 batch fermentation for high level production of its toxin proteins Cry4Ba and Cry11Aa was performed. As the interaction of the medium components as well as cultivation conditions are expected to influence the production of the toxin proteins, an experimental chart was prepared by accepting the previously reported optimal values for the most important parameters as zero points: [Mn], 10^{-6} M; [K_2HPO_4], 50 mM; C:N ratio, 20:1 and incubation temperature; 30°C. When the combinations of these variables at different levels were studied at 30 batch cultures and analysed for the optimum toxin protein concentrations, temperature: 28.3°C, [Mn]: $3.3x10^{-7}$M, C:N ratio: 22.2 and [K_2HPO_4]: 66.1mM yielded the highest concentrations of both Cry4Ba and Cry11Aa toxin proteins.

Key words: *Bacillus thuringiensis israelensis*, response surface optimization, delta endotoxin, crystal protein, Cry4Ba, Cry11Aa.

ÖZ

BACILLUS THURINGIENSIS ISRAELENSIS FERMENTASYONU İÇİN TEPKİ YÜZEY OPTİMİZASYONU

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December 2003, 86 sayfa

Günümüzde, pest populasyonunun böcek patojenleri kullanılarak kontrolü, aynı amaçla kimyasal pestisitlerin uygulamasına oldukça çekici bir alternatif oluşturmaktadır. Kimyasalların çevreye verdikleri zarar, pestlerin direnç geliştirmesi ve sadece hedeflenen organizmanın zarar görmeyip diğer canlıların da

etkilenmesi göz önüne alındığında, biyolojik mücadele en uygun çözümdür ve *Bacillus thuringiensis* (*Bt*) alttürleri tarım, ormancılık ve sivrisinek kontrolünde en çok kullanılan biyoinsektisitler olmuşlardır.

Bt türlerini diğer *Bacillus* türlerinden ayıran en önemli özellik, delta-endotoksin adı verilen protoksinler (kristal protein) sentezlemeleridir. Bu çalışmada, *Bt* alttür *israelensis* HD500 tarafından Cry4Ba ve Cry11Aa toksin proteinlerinin yüksek seviyede üretimi için kesikli fermentasyon tepki yüzey optimizasyonu ile incelenmiştir. Besiyeri bileşenlerinin birbiri ile olan etkileşimi ve kültivasyon koşulları toksin proteinlerinin sentezini etkilediğinden, daha önce rapor edilmiş en önemli parametrelerin optimal değerleri sıfır noktası kabul edilerek ([Mn], 10^{-6} M; [K_2HPO_4], 50 mM; C:N oranı, 20:1 ve sıcaklık, 30°C) bir deney çizelgesi hazırlanmıştır. Bu çizelgedeki değişkenlerin değişik seviyelerdeki kombinasyonları 30 ayrı kesikli kültürde çalışıldığında, sıcaklık 28.3°C, [Mn]: $3.3x10^{-7}$M, C:N oranı: 22.2 ve [K_2HPO_4]: 66.1mM, Cry4Ba ve Cry11Aa toksin proteinleri için en iyi verimi sağlayan değerler olarak bulunmuştur.

Anahtar Kelimeler: *Bacillus thuringiensis israelensis*, tepki yüzey optimizasyonu, delta-endotoksin, kristal protein, Cry4Ba, Cry11Aa.

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to Prof. Dr. Gülay Özcengiz and Prof. Dr. Gürdal Alaeddinoğlu for their guidance, advice and supervision of this study.

I am also grateful to Prof. Dr. Ülkü Mehmetoğlu and Assoc. Prof. Dr. Emine Bayraktar for their comments and guidance in this study.

I would like to thank to my labmates in Lab 13, our technicians Zeynel Akın and Hakan Şen for their help and corporation.

I would like to thank Hayriye Özçelik, Deniz Yücel, Halime Kenar, Oya Akçelik, Suzan Ber, Sibel Çınar, Şerife Ayaz, Süleyman Coşkun and Hande Erünlü for their encouragements, great friendship and patience.

I would like to thank Volkan Keskin for being with me any time I need with his endless support, encouragements, help, advice and patience.

I would like to thank my parents and brother for their endless love, support, patience and advice.

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LIST OF SYMBOLS

Bt	:	*Bacillus thuringiensis*
Bti	:	*Bacillus thuringiensis* subspecies *israelensis*
kDa	:	Kilodalton
MDa	:	Megadalton
mYSM	:	modified Yousten's Synthetic Medium
RSM	:	Response Surface Methodology
SDS-PAGE	:	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
YSM	:	Yousten's Synthetic Medium

CHAPTER 1

INTRODUCTION

1.1. Bacillus thuringiensis and Delta-Endotoxins (Crystal Proteins)

Bacillus thuringiensis (*Bt*) is a well known and widely studied bacterium which is known for its use in pest management. Today, it is the most successful commercial xenobiotic with its worldwide application. When compared with the chemical pesticides, *Bt* has the advantages of being biologically degradable, selectively active on pests and less likely to cause resistance. Safety of *Bt* formulations for humans, beneficial animals and plants explains the replacement of chemical pesticides in many countries with these environmentally friendly pest control agents.

Bt was first isolated by the Japanese scientist Ishiwata (1901) from silkworm larvae, *Bombyx mori*, exhibiting sotto disease. After 10 years, Berliner (1911) isolated the same Gram (+), spore-forming, rod shaped soil bacterium from diseased flour moth larvae, *Anagasta kuehniella*, in the Thuringia region of the Germany and named it as *Bacillus thuringiensis*.

In the early 1930s, *Bt* was used against *Ostrinia nubilis*, the European corn borer. The first commercial product was available in 1938 in France, with the trade name Sporeine (Weiser, 1986). It was *Bt* subspecies *kurstaki* that was used for the control of the insect (Lepidopteran) pests in agriculture and forestry (Lüthy and Ebersold, 1981). New commercial products arrived in 1980s after the discovery of subspecies *israelensis* in 1976 (Goldberg and Margalit, 1977). *Bt israelensis*, being a Diptera active subspecies, opened the gate for black fly and mosquito larvae control.

The larvicidal activity of *Bt* is attributed to parasporal crystals that it produces. Because of the crystalline structure of these crystals, they are called as crystal proteins (Cry) or delta endotoxins. The genes encoding for the crystal proteins are named as *cry* genes, and their common characteristic is the expression of the genes during the stationary phase. Therefore, the crystal proteins accumulate in the mother cell and are released upon completion of sporulation. Also a cytolytic protein, cytolysin (Cyt), is found in the crystal inclusions of the Diptera active strains.

Bt can be isolated from several different sources and different environments (Bernard, 1986; Martin and Travers, 1989; Smith and Couche, 1991). The natural strains can be classified according to their biochemical properties and flagellar (H) antigens (de Barjac and Fracon, 1990). To determine the pathotype of the strains,

this kind of a classification is not appropriate; instead delta endotoxin composition is the criterion that determines the range of insect pests on which the organism is active.

In 1989, Höfte and Whiteley proposed a systematic nomenclature and classified the crystal proteins in five major groups according to their insecticidal and molecular relationship (Cry I, Cry II, Cry III, Cry IV and Cry V, Cyt). As new strains are discovered, a need for a new nomenclature arose. According to new nomenclature which is used today (Crickmore *et al.*, 1998), roman numerals have been exchanged with the Arabic numerals and the strains are named on the basis of their evolutionary divergence. Additionally, underneath the capital letters which were present at the first nomenclature as well, small letters have been brought indicating the minor amino acid differences like the capital letters denoted for the major differences. As a result of this classification, today there are as much as 40 major Cry protein classes. The gene for the 40^{th} crystal protein was recently cloned from *Bt aizawai* with mosquitocidal activity (Ito *et al*., 2003). It is also to be noted that most *Bt* strains produce more than one type of crystal protein that act in combination.

When the structural and sequential similarities are considered, conserved amino acid sequences drew attention among most of the Cry toxins (Höfte and Whiteley, 1989). According to these similarities and insecticidal activities, the properties of

the Cry proteins differ and the members of the same group share a number of common features. Table 1.1 shows the types of crystal proteins and the insect orders to which they are active.

Table1.1. The Cry protein groups and the orders they are pathogenic for.

Lepidopteran larvicidal	Cry1, Cry9, Cry15
Coleopteran larvicidal	Cry3, Cry7, Cry8, Cry14, Cry34, Cry35, Cry36, Cry38
Dipteran larvicidal	Cry4, Cry10, Cry11, Cry16 (Cry17), Cry19, Cry20, Cry24, Cry25, Cry27, Cry29, Cry30, Cry39, Cry40
Lepidopteran and Dipteran larvicidal	Cry2
Nematicidal	Cry5, Cry6, Cry12, Cry13, Cry21
Active on Hymenopteran	Cry22

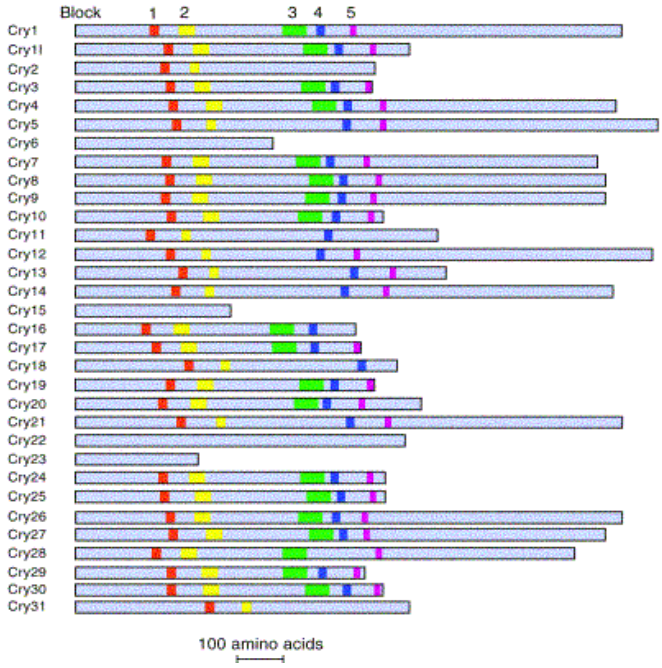


Figure 1.1. Conserved sequences of insecticidal crystal proteins.

Shown in Figure 1.1 are the 5 common conserved regions of the Cry proteins. Domain I of the proteins starts from the N-terminal and includes the conserved region 1, the conserved block 2 lays between the 2nd and 3rd domains. The second domain lays down to the conserved region 3 which is the amino acid sequence between the domain II and domain III. The amino acid sequence which starts by the end of the conserved block 5 and continues down to the C-terminal is the part cleaved by the proteases during crystal protein activation (de Maagd *et al*., 2001). All widely known crystal toxins and the remaining homologues of these proteins are indeed protoxins, which are not active when they are synthesized. Only the Cyt toxin is active in its native form. To become active, all other protoxins must be cleaved by proteases. There are eight distinct consensus sequences in the

structure of crystal protoxins. These consensus sequences determine the similarity groups (Schnepf *et al*., 1998). Yet, the five conserved sequences shown in Figure 1.1 are the most common of these blocks (de Maagd, 2001). Within different classes of crystal proteins, the number of the common blocks can also differ. Cry1, 3, 4, 7, 8, 9, 10, 16, 17, 19 and 20 have five blocks of these consensus sequences, while Cry5, 12, 13, 14 and 21 contains four of the homologue blocks. When compared with the first group, the group involving Cry5 has more variability when the conserved sequences are compared within the groups and between the groups (Schnepf *et al.,* 1998). Lastly, Cyt1, 2, Cry6, 15 and 22 have no recognizable homologues of these blocks.

The crystal proteins are synthesized as protoxins that are not active. Protoxins possess three domains. Each domain has a different structure and function. Domain III contains the cleavage site for the proteases, domain II has the receptor binding capacity determining the species specificity as well as the domain III and finally domain I has the membrane insertion capacity for the toxic action in target organism. Domain I is composed of several alpha helices which are hydrophobic, making the membrane insertion possible (Hodgman and Ellar, 1990; Li *et al.*, 1991; Duche *et al.,* 1994; Lesieru *et al.,* 1997). The beta-sheet structure of domain II shows similarities with the antigen binding sites of immunoglobulins and it is known to recognize the carbohydrate moieties of its receptors (Knowles *et al.,* 1984; Schnepf *et al.,* 1998). Like domain II, domain III is also composed of beta-sheets, but it forms a beta sandwich structure. It maintains the structural integrity

of the toxin molecule and protects the protein from cleavage by the proteases in the gut of the target organism (Li *et al.*, 1991; Schnepf *et al.,* 1998). Figure 1.2 illustrates the 3D structure and the domains of the crystal proteins.

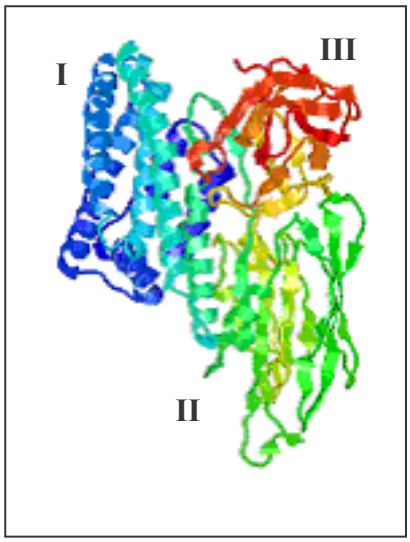


Figure 1.2. 3D structure and the domains of crystal protein Cry1A

The mechanism of action of the Cry proteins starts with the binding of the protein to the midgut epithelial cell receptors, but before this, solubilization of the inclusions at the reducing conditions is required (Schnepf *et al.,* 1998). This reducing condition is present at the alkaline environment of the insect midgut with a pH value of 10 (Hofman *et al.,* 1988). After solubilization, the inactive protoxin must be activated through proteolytic cleavage by the insect midgut proteases (Lecadet and Dedonder, 1967; Tojo and Aizawa, 1983). The cleavage takes place at the C-terminus end of the protein (Choma *et al.,* 1991). In the case of Cry1A

protoxins, the cleavage is performed by the chymotrypsin-like or trypsin-like proteases (Lecadet and Dedonder, 1966, 1967; Johnston *et al.,* 1995; Peterson *et al.,* 1995; Novillo *et al.,* 1997). The initial molecular weight of the protein decreases from 130 kDa to 65 kDa via clipping off 10 kDa sections (Choma *et al.,* 1991).

Activated Cry toxin then binds to specific receptors on the apical brush border of the midgut microvilliae of the insect (Schnepf *et al.,* 1998). In several insects, there are aminopeptidase enzymes which may possess more than one binding site (Masson *et al.,* 1995; Cooper *et al.,* 1998; Jenkins *et al*., 2000). Found also are cadherin-like proteins functioning as receptors for Cry toxins (Nagamatsu *et al.*, 1998, 1999; Gahan *et al.,* 2001; Griffitts *et al.*, 2001; Dorsch *et al.,* 2002; Hirotaka *et al.,* 2003). Binding has two stages, reversible binding to the receptor and irreversible binding that involves the insertion of the toxin into the membrane. The reversible binding is performed by binding of domain II to the receptor. This binding is very important for the further activity of the toxin, because loose bindings can increase dissociation which can decrease the toxicity. Also, if the binding affinity is higher, the toxicity can be greater (Schnepf *et al.,* 1998). Binding of domain II to the receptor brings the first domain closer to the membrane. When domain I comes closer to the membrane, it becomes inserted into the membrane, which is called the irreversible binding. This irreversible binding has not yet been well understood. There are two proposed mechanisms to explain it. The first model, namely the umbrella model, supports a hairpin

formation. Accordingly, two hydrophobic alpha helices (α8- α9) are inserted into the membrane after a pH-induced conformational change followed by pore formation (Duche *et al.,* 1994; Lesieru *et al.,* 1997). This model also proposed a similar hairpin insertion causing pore formation, but with different alpha helices (α4- α5) of domain I (Li *et al.*, 1991) which was later supported by the mutation experiments of Schwartz *et al.* (1997). The other model is called the pen–knife model (Hodgman and Ellar, 1990). In this model, the alpha helices (α5- α6) which are also hydrophobic are inserted into the membrane, but this time they are joined to form a loop which remains on the top of the membrane or attached to the receptor.

After insertion into the membrane and pore formation, an influx of the water with the ions occurs that causes to cells swelling and finally lead to their lysis (Knowles and Ellar, 1987). The lysis of the cells due to the formation of nonspecific pores causes the paralysis of the gut and the larvae stop feeding, this brings about larval death (Schwartz *et al.,* 1993; Lorence *et al.*, 1995; Pietrantonio and Gill, 1996). Figure 1.3 illustrates pore formation by *Bt* toxins.

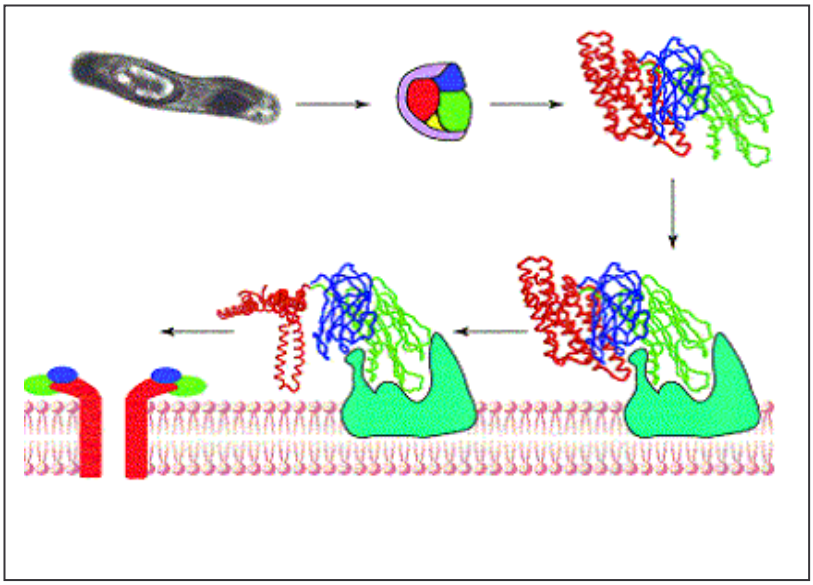


Figure 1.3. Pore formation by *Bt* crystal proteins

The resistance to the Cry proteins arises because of the reduced binding of the proteins to the receptors, reduced protoxin activation and increased toxin degradation (Schnepf et al., 1998). The resistance trait was shown to be recessive (Schnepf *et al*., 1998; Marroquin *et al*., 2000) and if different Cry proteins were used in combination for the pest control, the sensitivity to the toxins could be recovered. Georghiou and Wirth (1997) reported that *Culex quinqefasciatus* (mosquito) developed resistance to Cry11A toxin itself, but when the other crystal proteins that are synthesized by *Bt israelensis* were used in combination, the mosquitoes remained sensitive to the toxin activity. Wirth *et al*. (2003) reported that Cyt1A synthesized by *Bt israelensis* was an important component that

suppresses resistance or delays its development. Another study conducted by Marroquin *et al*. (2000) has shown that the nematodes resistant to Cry5B delta-endotoxin were susceptible to another Cry protein, Cry6A.

The insecticidal crystal proteins of *Bt* are known to be encoded by large plasmids and *cry* gene sequences homologues to the ones in plasmids also occur in chromosomes (Gonzalez *et al*., 1981; Kamdar *et al*., 1983, Honigman *et al*., 1986; Carlson and Kolsto, 1993). The *cry* genes were also sequenced between transposons and insertion sequences (Schnepf *et al*., 1998). By the help of these mobile elements, the genes are transferred via transduction, transformation, but most importantly by direct plasmid transfer via conjugation (Sekar, 1990). By taking this information as a base, the cloning of the crystal toxin genes have been achieved by many laboratories (Schnepf and Whiteley, 1981; Delecluse *et al*., 1988; Bar *et al*., 1990; Donovan *et al*., 1992). The *cry2Aa* gene from *Bt kurstaki* has been expressed in an endophytic isolate of *Bacillus cereus* (Mahaffee *et al.*, 1994). Alfalfa and pea were protected from the coleopteran pests, clover root cuculio (*Sitona hispidulus*) and pea leaf weevil (*Sitona lineatus*) upon transfer of the *cry3Aa* gene from *Bt tenebrionis* to *Rhizobium melloti* and *Rhizobium leguminosarum*, respectively (Bezdicek *et al.*, 1994). Giddings *et al.* (1997) have reported a better nodule occupency by *R. leguminosarum* carrying the *cry3Aa* gene. Baculoviruses (e.g. *Autographa californica*), the naturally pest occurring pathogens have also been employed as an expression system for *cry* genes (Martens *et al.*, 1995; Je *et al*., 1997). As more knowledge gained in *Bt* genetics,

the new strain improvement strategies have been developed, the purpose being to increase the toxicity to the pests, to broaden the range of target pests and to delay the onset of resistance (Kaur, 2000). Sun *et al*. (2001) has reported the reduction of resistance of *Culex pipiens* larvae when they are exposed to the *Bt israelensis* producing both the Cry4Ba and the binary toxin of *Bacillus sphaericus*, as these proteins have different receptors and different action mechanisms. The transfer of crystal protein genes to alternative delivery systems is also an important approach. The persistence of crystal proteins in the environment is limited due to UV, heat and pH (Kaur, 2000). An important delivery system was the encapsulation of the cry genes into the non-pathogenic *Pseudomonas fluorescens* cells. These cells were killed by a proper chemical treatment after fermentation which made the bacterial cell wall more rigid, moreover the control was found to be more safe and more effective than the conventional *Bt* spraying because the cells were not alive and the crystals were protected by the cell wall of the dead microorganisms (Gaertner *et al.*, 1993; Carlton, 1996). Nambiar *et al*. (1990) cloned the delta-endotoxin gene from *Bt israelensis* to a plasmid vector which was then transferred to a *Bradyrhizobium* species that nodulates pigeon pea and used for controlling the dipteran (*Rivellia angulatum*) larval attack. The same transfer approach was taken by Lima *et al*. (2000), but resulted with the low expression of the *cry1A(a)* gene from *Bt kurstaki*-HD1. Tanapongpipat *et al*. (2003) have integrated the mosquitocidal genes of *Bt israelensis* and *Bacillus sphaericus* into the chromosome of *Enterobacter amnigenus*, the latter organism was isolated from

the guts of *Anopheles dirus*, and it was able to recolonize and persist in the guts of *Anopheles* larvae. The investigators showed that the integrants were expressed at very low levels, but were stable for long periods.

The *cry* genes have been introduced into several plants. The first plant to express these genes was tobacco (Vaeck *et al*., 1987; Barton *et al*., 1987). Later on, potato, cotton, rice, corn were transformed and at 1996, transgenic potato, cotton and corn carrying *cry* genes were sold to farmers (Schnepf *et al*., 1998). As these crops provide effective control of major insect pests, farmers rapidly adopted the Bt-crops to provide higher yields (Betz *et al*., 2000). The higher yields of rice have been obtained in Asia by the use of Bt-rice (Toenniessen *et al*., 2003). The use of Bt-crops also reduces insecticide use and costs (Betz *et al*., 2000; Toenniessen *et al*., 2003). Although there is a controversy about the use of Bt-plants due to possible adverse effects to mammals and development of resistance by the pests, South Africa, Argentina, North America and Europe grow Bt-corn. The reason for high fumonisin levels in native corn is its high susceptibility to corn borers that introduce damaged plant tissues promoting fungal (*Fusarium*) growth and mycotoxin production. Munkvold *et al*. (1997, 1999) and Dowd (2000) have reported that the Bt-corn present 30-40 folds lower levels of fumonisin, than the native corn in Illinois. It has been reported that fumonisins were toxic to animals and humans, causing cancer and death respectively (Marasas *et al*., 1988, Norred *et al*., 1993). As diamondback moth developed resistance due to intensive spraying of the crops (Tabashnik, 1994). Because of

the widespread planting of Bt-crops in 1999, there arose the probability that more insect species could develop resistance to crystal proteins (Cerda and Wright, 2002). To prevent development of resistance in 2000, US Environmental Protection Agency has announced the necessity of planting of at least 20% non Bt-corn and 50% non Bt-cotton. In 2001, 20% of the global cotton acres were plated with transgenic Bt-cotton (Carpenter *et al*., 2002).

Apart from transgenic Bt-plants, *Bt* formulations themselves are also very important in plant protection. These formulations are also used for the black fly and mosquito control. The formulations can include wetting agents, stickers, sunscreeners, synergists and phagostimulants (Navon, 2000). It is well known that crystal proteins are very sensitive to UV and UV also is the major cause for rapid *Bt* inactivition especially between the wavelength range of 250 to 380 nm (Ignoffo *et al*., 1981; Pozsgay *et al*., 1987; Navon, 2000). To protect the formulations from UV light, Liu *et al*. (1993) has developed a process. By the fermentation of *Streptomyces lividans* 66 containing a recombinant plasmid which encoded for the melanin production, high amounts of melanin were obtained. When the melanin was added into the *Bt israelensis* formulation, it was seen that at wavelengths ranging from 210 to 280 nm, melanin provided an excellent photoprotection. This recombinant *Bt* producing melanin also provided a higher insecticidal activity (Patel *et al*., 1996). Other approaches for UV protection have involved the addition of chromophores to the formulations such as methyl green and acriflavin (Cohen *et al*., 1991; Margulies *et al*., 1985), but their use is risky when

environmental safety is considered (Navon, 2000). To reduce washing of the *Bt* formulations, biopolymers for *Bt* complex encapsulation have been developed (Ramos *et al*., 1998). Another approach for the development of the *Bt* formulations is the addition of phagostimulants, as larvae feed selectively. The larvae will be attracted to the *Bt* product and will tend to feed less on the plants or other sources if phagostimulants are present (Navon, 2000). For black fly and mosquito control, the efficacy of the formulation also depends on the stability of the formulation on the water surface. Liquid formulations are being replaced by dry formulations as they sustain more on the water surface (Couch, 2000). In a study conducted by Gunasekaran *et al*. (2002), an alginate-based sustained release formulation of *Bti* was tested against *Culex quinquefasciatus* and proved successful. Moreover, the study provided 80% better control of pupae when compared with a similar study (Apperson *et al*., 1986) performed by using a commercially available *Bt* formulation, namely Bactimos.

To develop a *Bt* formulation, it is important to cultivate the bacterial strain to obtain the highest yields of spore-crystal complexes. In general, the nutritional and cultural requirements vary with the *Bt* strain. The parameters optimized for one strain never work well for another strain. The culture conditions for *Bt* must be optimized to give high cell yield, high crystal protein concentration and high toxicity (Dulmage *et al*., 1990 a). Previously, it was believed to be important to end up with high spore counts, but it was later shown that high spore counts do not always mean high toxicity (Rosa and Mignone, 1993; Paramatha, 2000).

For the fermentation of *Bt*, carbohydrates are very important. However, when used at high concentrations, they can cause adverse effects because *Bt* produces acids from carbohydrates. The utilization of carbohydrates to produce acidic forms can decrease the pH below 5.5 – 5.7 range at which most *Bt* strains can not grow, thus the fermentation stops (Dulmage *et al*., 1990a). To prevent such conditions, it is important to maintain a balance between carbon and nitrogen ratio and/or the pH is controlled by adding alkali when required during the process. Farrera *et al*. (1998) has shown that C:N ratio balance itself was directly important for the crystal protein production. They have investigated the range of 3:1 to 11:1 C:N ratio for the fermentation of *Bt kurstaki* HD-73 and although the spore count was the highest with 4:1 ratio, the crystal protein concentration was highest when C:N ratio was 7:1. A controversy does also exist about the role of glucose in *Bt* fermentations. Scherrer *et al*. (1973) showed that when the glucose concentration in the medium was increased, the size and potency of the crystal proteins also increased. However, glucose is also known to repress several operons and genes in Gram-positive bacteria through carbon catabolite repression. Bhatnagar (1998) showed that Cry4A toxin of *Bt israelensis* was not synthesized when the medium contained 0.4% glucose. The mRNA levels were very low when glucose was at that concentration when compared to the glucose depleted medium at the same hours of fermentation.

Another important component for the production of crystal protein was potassium. Wakisaka *et al*. (1982) cultivated *Bt* in a media containing several potassium salts

and detected an increased crystal protein production. However, when potassium salts were replaced with the same salts of sodium, the same effect was not seen. A similar response was also reported by Foda *et al*. (1985).

Other important components for the production of crystal proteins are the trace minerals (Rose, 1979). Sikdar *et al*. (1991) found that Fe, Mn and Cu were required for the production of crystal protein while Mo had an inhibitory effect.

Sachinanandham *et al*. (1997) demonstrated that several amino acids resulted in better carbon utilization and also improved the stability and volumetric productivity of biomass for *Bt galleriae*. Moreover, when amino acids were supplied to the medium, the formation of spores and crystal proteins improved.

In our laboratory, the regulation of crystal protein biosynthesis has been investigated by İçgen *et al*. (2002a, b) and Özkan *et al*. (2003). The two former reports focused on crystal protein biosynthesis by an antilepidopteran strain of *Bt*, isolated locally and designated *Bt* 81. The most important metals for the synthesis of crystal proteins were found to be Mg and Cu. Mg was found to be essential for the synthesis of the crystal proteins as the level of crystal protein synthesis was almost zero when Mg was omitted from the medium. The stimulatory effect of Cu was seen between the range of 10^{-6} to 10^{-7} M. Ca and Zn had no effect on toxin production while Mn favored the crystal protein synthesis in a range of 3×10^{-4} to 10^{-5} M. Co-regulation of sporulation and crystal protein synthesis by the minerals

was not evident. Also, the suppression of crystal protein biosynthesis by inorganic phosphate over a range of 3 to 100 mM, as a general suggestion for secondary metabolism (Demain, 1982) was not seen. The highest toxin concentrations were obtained when sucrose, lactose or inulin was used as carbon sources. Glucose, glycerol, maltose starch and dextrin on the other hand, yielded low crystal protein concentrations. Of various organic and inorganic nitrogen sources investigated, peptone was found to be the best.

Özkan *et al*. (2003) investigated the optimal parameters for the production of antidipteran crystal proteins from *Bt israelensis* HD500. According to this study, Fe, Zn and Cu negatively influenced the synthesis of the crystal proteins. Mg and Ca favored the toxin production while Mn was found to be the most critical trace element. For the production of crystal proteins, high concentrations of inorganic phosphate were found to be beneficial. As to the carbon sources, sucrose, lactose, whey, glycerol, dextrin, maltose or inulin were found to be stimulatory, while starch, glucose or molasses were suppressive.

Several studies were conducted to decrease the cost of fermentation by employing low-cost media components. For example, Poopathi *et al*. (2002) suggests efficient use of a potato-based culture media for the industrial production of *Bt israelensis*. Vora and Shetna (1999) reported enhanced growth, sporulation and toxin yields by *Bt kurstaki* when defatted soybean and groundnut seed meal extracts were used with the supplementation of cystine. Gruel and fish meal media

were also investigated for the growth of both Diptera active and Lepidoptera active *Bt* strains and their use to cultivate antilepidopteran subspecies was found to be more beneficial than their use for the antidipteran supspecies which displayed decreased toxicity (Zouari *et al*., 2002). In recent years, wastewater sludge as a raw material for the production of *Bt kurstaki* was also evaluated (Lachhab *et al*., 2001; Montiel *et al*., 2001; Vidyarthi *et al*., 2002).

1.2. *Bacillus thuringiensis israelensis* for Mosquito and Black Fly Control

Bt israelensis (*Bti*) was first isolated by Goldberg and Margalit (1977) in Israel, from dead *Culex pipiens* larvae and the isolate was named as ONR60. This strain was identified as *Bti* H14 by de Barjac (1978). As it has antidipteran activity, *Bti* is very important in the control of mosquito- and black fly-borne human diseases.

The larvicidal activity of *Bti* is due to the presence of *cry4A, cry4B, cry10A, cry11A* and *cyt1A* genes. All these genes can either be present in combination in some strains or some can be absent from the others, yet they act synergistically in all probabilities (Schnepf *et al*., 1998). The large 72 MDa plasmid is known to carry these gene sequences which can also be transferred by a conjugation-like process (Gonzalez *et al*., 1982). There are also several subspecies that carry antidipteran Cry proteins that can be identical to or different than the ones present in *Bti* (Padua *et al*., 1980). Table 1 presents a list of antidipteran Cry proteins.

The activity of *Bti* is influenced by varying environmental and biological factors (Lacey, 1985). The biological factors are mainly the feeding behaviour of the larvae, instar-related sensitivity and larval density. The environmental factors include flowing water, water depth, presence of organic extraneous materials in the water, vegetative cover on the water surface and low water temperature (Mulla, 1990; Molloy, 1990).

Mosquito control by using *Bti* was also made in Germany in 1976 for the management of Upper Rhine province. The German Mosquito Control Association (GMCA/KABS) has conducted this study for more than 15 years and for the control of both *Culex pipiens* and *Aedes vexans* larvae. Culnex®-Bti tablets were used together with *Bacillus sphaericus* applications. This approach provided the conservation of the biodiversity through the Rhine Valley as well (Becker, 1997).

In tropical countries, the first field trials of *Bti* were carried out to control mosquitoes and black flies to reduce the risk of mosquito and black fly-borne diseases, in 1980s (da Regis, 2001). It was also employed to control a black fly borne disease, namely onchocerciasis for 16 years, in 11 countries of West Africa as a part of long-term public health programme (Hougard *et al*., 1997; Molyneux, 1995). Such control programmes were also conducted in Brazil to replace the previously used chemicals that evoked resistance (Mardini *et al*., 1999).

In the studies of Chinese work groups, *Bti* was shown to decrease the incidence of malaria significantly (Becker, 1997). Kroeger *et al*. (1995) conducted a study about the efficacy of the *Bti* spraying in mosquito breeding places in the Pacific coast of Peru and Equador and in the Amazon area of Peru. In these areas, *Bti* was applied weekly for more than 7 weeks. It was shown that the *Anopheles* adult density was reduced by 50-70 %. This study indicates the potential of *Bti* as a malaria control agent.

1.3. Response Surface Methodology (RSM) and the Aim of the Present Study

The most extensive applications of RSM are in the industrial world, particularly in situations where several input variables potentially influence some performance measure or quality characteristic of the product or process. This performance measure or quality characteristic is called the response. It is typically measured on a continuous scale, although attribute responses, ranks and sensory responses are not unusual. Most real-world applications of RSM will involve more than one response. Most applications of RSM are sequential in nature. That is, at first some ideas are generated concerning which factors or variables are likely to be important in the response surface study. This usually leads to an experiment designed to investigate these factors with a view toward eliminating the unimportant ones. This type of experiment is usually called a screening experiment. Often at the outset of a response surface study there is a rather long list of variables that could be important in explaining the response. The objective

of factor screening is to reduce this list of candidate variables to a relatively few so that subsequent experiments will be more efficient and require fewer runs to test (Khuri and Cornell, 1996; Myers and Montgomery, 2002).

Our earlier studies involving protein profile analysis of sporulated cultures of different *Bti* strains (HD500, 4Q2-72 and HD14) revealed that Cry4Ba, Cry11Aa and Cyt1A toxin components were common to all of the strains while Cry4A and Cry4C proteins could not be detected in any of them. The effects of various nutritional and cultural factors for *Bt* subsp. *israelensis* HD500 crystal toxin components Cry4Ba and Cry11Aa have been reported by our laboratory (Özkan *et al.,* 2003). On the basis of these findings, and the C:N ratio effect demonstrated earlier the different fermentation conditions affecting the crystal protein synthesis is aimed to be investigated in this study by RSM.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial Strains and Their Maintenance

The strains of *Bacillus thuringiensis israelensis* (*Bti*) used in this study were *Bti* HD500 and ONR60.

The strains were streaked on nutrient agar and subcultured monthly. They were stored at 4°C. For long term maintenance of the strains, bacterial stocks were prepared by using a method adopted from the storage procedure described by Stahly *et al.* (1992):

Bti HD500 was grown in 25 mL of modified YSM (mYSM) to mid-log phase (A_{600} = 0.5). A 100 µL aliquot from this culture was diluted 50 folds by adding 4.9 mL of 50 % glycerol (v/v). The diluted culture was then divided in Eppendorf tubes as 100 µL aliquots and stored at – 70°C. To run fresh cultures from such

frozen samples, a 50 µL aliquot was inoculated into 25 mL of mYSM and grown by shaking. When the culture reached to mid-log phase, a required amount from this culture (seed culture) was transferred to fresh mYSM.

2.2. Culture Media

The Yousten's Synthetic Medium (YSM) which was modified by Özkan *et al.* (1997, 2003) was used. The composition and preparation of this medium is given in Appendix A.

2.3. Buffers

The buffers and their composition are listed in Appendix B.

2.4. Chemicals

The chemicals used and their suppliers are listed in Appendix C.

2.5. Growth Curves

500 µL of an overnight culture was used to inoculate 50 mL of mYSM in a 250 mL Erlenmayer flask. The culture was incubated at 30°C by shaking for 72 hrs at 200 rpm. Inoculation time was considered as time zero. Samples were taken from

the culture with 2 h intervals and used for quantitative determination of growth which was measured spectrophotometrically at 600 nm. Viable counts were determined as “colony forming units/mL” for both the strains simultaneously.

2.6. Protein Extraction

A slightly modified procedure of Armelle (1991) was used for protein extraction. 10 mL of 48 h bacterial cultures were centrifuged at 4 000 rpm for 10 min. The pellet was resuspended in 500 µL of 1 M NaCl. This mixture was transferred into an Eppendorf tube and centrifuged at 7000 rpm for 7 min. The pellet was resuspended in 250 µL of TE buffer (Appendix B) and centrifuged at 7000 rpm for 7 min. Then, the pellet was suspended in 250 µL of dH_2O and centrifuged at 7000 rpm for 7 min. After discarding the supernatant, 150 µL of 10 mg/mL lysozyme solution in TE buffer was added and the suspension was incubated at 37°C for 30 min. 25 µL of 10% SDS solution was added into the suspension which was then Vortexed for 30 sec. It was centrifuged at 6000 rpm for 10 min and 100 µL of 0.2% SDS solution was added to the pellet. For denaturation, 60 µL of gel loading buffer (Appendix B) was added to 30 µL of this mixture in another Eppendorf tube. Finally, this sample was incubated at 90°C in a water bath for 7 min for three times and placed on ice until it cools. The final native and denatured samples were stored at -20°C.

Protein concentrations in the denatured samples were adjusted to the same level prior to gel electrophoresis. The protein concentrations of the samples were determined by Bradford method.

10 µL of native sample was made up to 0.5 mL with dH_2O and Vortexed after the final volume was brought to 5 mL with Bradford reagent (Appendix B). After 10 min, the mixture was Vortexed again and the absorbance was measured at 595 nm. Concentrations of the samples were calculated from the calibration curve which was also prepared at each batch of the experiments. 25 µL of each sample was applied to gel after equalizing protein concentration.

2.7. SDS-Polyacrylamide Gel Electrophoresis

The proteins were separated by using a vertical polyacrylamide gel apparatus. Electrophoresis was run at 20 mA at the beginning when the samples reached to the separating gel, then the current was increased to 40 mA and the electrophoresis was continued until the samples reached to the end of the gel.

The SDS-polyacrylamide gels were prepared according to the description below:

	Stacking Gel **(4.5 %)**	*Separating Gel* **(10 %)**
45 % Acrylamide	2 mL	9 mL
Stacking buffer (Appendix B)	5 mL	12 mL
dH_2O	12 mL	24 mL
Ammonium persulfate	5 mg	20 mg
10 % SDS	200 µL	450 µL
TEMED	25 µL	50 µL

Protein molecular weight markers (Fermentas) were as follows:

Beta-galactosidase (116 kDa), Bovine serum albumin (66.2 kDa), Ovalbumin (45 kDa), Lactate dehydrogenase (35 kDa), RE *Bsp*98I (25 kDa), beta- globulin (18.4 kDa) and Lysozyme (14.4 kDa).

2.8. Staining of SDS-Polyacrylamide Gel

Staining of SDS- polyacrylamide gels was performed as follows (Blum *et al.*, 1987):

Step	*Solution*	*Time of treatment*
Fixation	50 % Methanol 12 % Acetic acid 0.05 % Formaldehyde	o/n
Washing	50 % Ethanol	3 x 20 min.
Pretreatment	0.29 g/L $Na_2S_2O_3$	1 min
Rinse	dH_2O	2 x 20 sec.
Impregnate	2 g/L $AgNO_3$ 0.75 mL/L Formaldehyde	20 min.
Rinse	dH_2O	2 x 20 sec.
Development	60 g/L Na_2CO_3 0.5 mL/L Formaldehyde 4 mg/L $Na_2S_2O_3$	1 – 5 min.
Rinse	dH_2O	2 x 20 sec.
Stop	50 % Methanol 12 % Acetic acid	
Store	50 % Methanol	

2.9.Response Surface Optimization

Response surface optimization was performed by the Design Expert 6.0 Software; (www.statease.com.) 30 experiments were carried out. The conditions in 24 sets of these 30 experiments involved the combinations of the coded values of the

experimental variables and the remaining 6 represented the central points. The test variables were coded according to the following equation:

$$x_i = \frac{(X_i - X_i^*)}{\Delta X_i} \qquad \text{(Eq 2.1)}$$

where x_i is the coded value of the i-th independent variable, X_i is the uncoded value of the i-th independent variable, X_i^* is the uncoded i-th independent variable at the center point and ΔX_i is the step change value. More specifically, coded values of the independent variables were calculated as follows:

$$x_1 = \frac{(X_1 - (-6))}{1} \qquad \text{(Eq 2.2)}$$

$$x_2 = \frac{(X_2 - 30)}{2.5} \qquad \text{(Eq 2.3)}$$

$$x_3 = \frac{(X_3 - 20)}{5} \qquad \text{(Eq 2.4)}$$

$$x_4 = \frac{(X_4 - 50)}{25} \qquad \text{(Eq 2.5)}$$

2.10. Protein Quantification

Gels were photographed by the Vilber Gel Imaging system and the amount protein in the bands corresponding to Cry4Ba and Cry11Aa proteins were determined by using Bio1D version 99 (Wilber-Lourmat).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Comparison of Toxin Production Capacities of *Bacillus thuringiensis israelensis* HD500 and ONR60 Strains as the Functions of Carbon Source, C:N Ratio and Incubation Time

As mentioned earlier, the crystals of *Bt* subsp. *israelensis* contain four major endotoxins designated Cry4A (125 kDa), Cry4B (134 kDa), Cry11A (67 kDa) and Cyt1A (27 kDa). No single crystal protein is as toxic as the intact crystal complex (Höfte and Whitely, 1989; Crickmore *et al.*, 1998). These protein toxins are expressed concominant with sporulation. Environmental factors play a critical role in modulating the differentiation pattern and synthesis of toxins which form a distinct group of secondary metabolites; thus their synthesis has considerably narrower tolerances for concentrations of specific trace metals and inorganic phosphate, as well as for ranges of temperature, pH and redox potential than does growth of the producer cells (Weinberg, 1974; Bhatnagar, 1998). Also, the repression or inhibition of secondary metabolism by catabolism of rapidly utilized

carbon sources, especially glucose (carbon catabolite regulation) and nitrogen sources, especially ammonium (nitrogen catabolite regulation) has been frequently reported and well illustrated (Demain, 1995).

Although the structure of the *Bt* toxins, their action mechanisms, molecular biology and genetics of toxin biosynthesis are well documented, there are relatively a few literature reports about its fermentation and industrial production. The importance of the medium components and other variables required during fermentation process is well known (Farrera *et al.*, 1998). Fermentation studies by using conventional methods (changing one variable at a time and keeping the others constant) takes a long time, moreover this kind of a study does not represent the interaction of the medium components with each other (Cohran and Cox, 1992). In that sense, when compared with the conventional methods, response surface methodology is a time saving method which also reveals the interaction between the components of a medium (Adinarayana and Ellaiah, 2002).

Our earlier studies involving protein profile analysis of sporulated cultures of different *Bti* strains (HD500, 4Q2-72 and HD14) revealed that Cry4Ba, Cry11Aa and Cyt1A toxin components were common to all of the strains while Cry4A and Cry4C proteins could not be detected in any of them. The effects of various nutritional and cultural factors for *Bt* subsp. *israelensis* HD500 crystal toxin

components Cry4Ba and Cry11Aa have been reported by our laboratory (Özkan *et al.,* 2003). On the basis of these findings, *Bacillus thuringiensis israelensis* HD500 were initially grown in two different carbon sources(sucrose and dextrin) with varying C:N ratios. Based on these results, ONR60 (known to be a highly toxic strain of *Bti*) (Margalit, 1990) were grown later under the same set of cultural conditions and a comparison were made between the two strains.

Bt subspecies produce acid from carbohydrates like many bacteria. This is why a balance between acid forming carbohydrates and the alkali-forming nutrient sources must be made properly (Dulmage *et al*., 1990b). In the case of higher amounts of carbohydrate present, the pH of the medium can drop below 5.5 which would than be intolerable for further cell growth. To avoid such detrimental changes in pH, alkali can be added to the medium regularly or alternatively the nitrogen content of the medium is increased, favouring the formation of alkaline products (Dulmage *et al*., 1990 a, b). A proper C:N ratio is therefore necessary in cultural conditions.

In order to compare different C:N ratios (4:1, 7:1, 10:1, 20:1 and 30:1) in the same series of experiments, parallel cultures of HD500 were run for 48^{th} h. As seen in Table 3.1, the C:N ratio of 20:1 yielded highest amount of Cry4Ba toxin and fairly good amount of Cry11Aa component.

Table 3.1. Concentrations of Cry4Ba and Cry11Aa at different C:N ratios with sucrose for *Bti* HD500.

	Crystal protein concentrations (mg/mL)				
	4:1	7:1	10:1	20:1	30:1
Cry4Ba	0.0024	0.0011	0.0035	0.0081	0.0073
Cry11Aa	0.0141	0.0088	0.0105	0.0092	0.0094

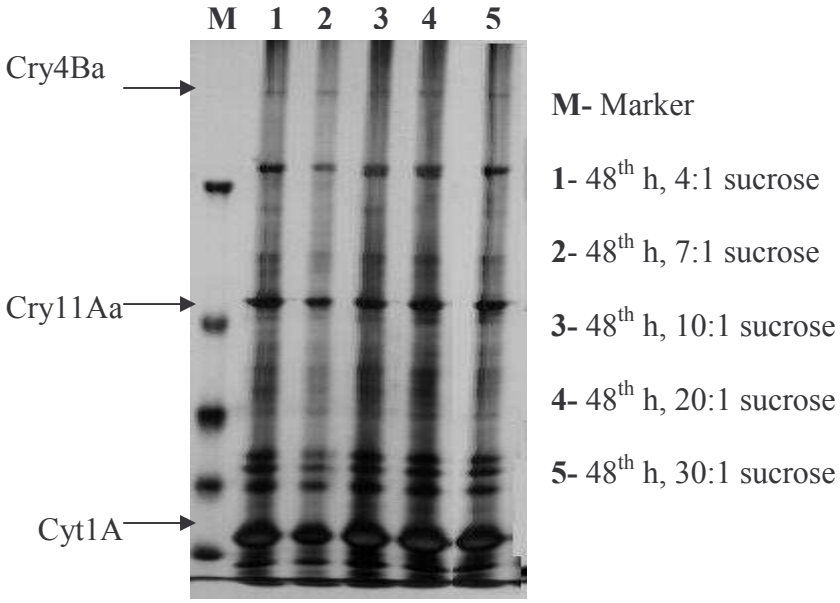


Figure 3.1. Effect of C:N ratios of 4:1, 7:1, 10:1, 20:1 and 30:1 with sucrose on the production of crystal proteins of *Bti* HD500.

Similarly, the analysis of the protein bands indicated that the C:N ratio of 20:1 was most appropriate for Cry4Ba which is known to be more important in toxicity than Cry11Aa (Federici *et al.*, 1990).

For comparison, the growth of *Bti* HD500 and *Bti* ONR60 in the mYSM having a C:N ratio of 20:1 was initially monitored against time. The results indicated that there was almost no difference between the growth rates and toxin yields of the strains. (Figure 3.2 and 3.3 respectively).

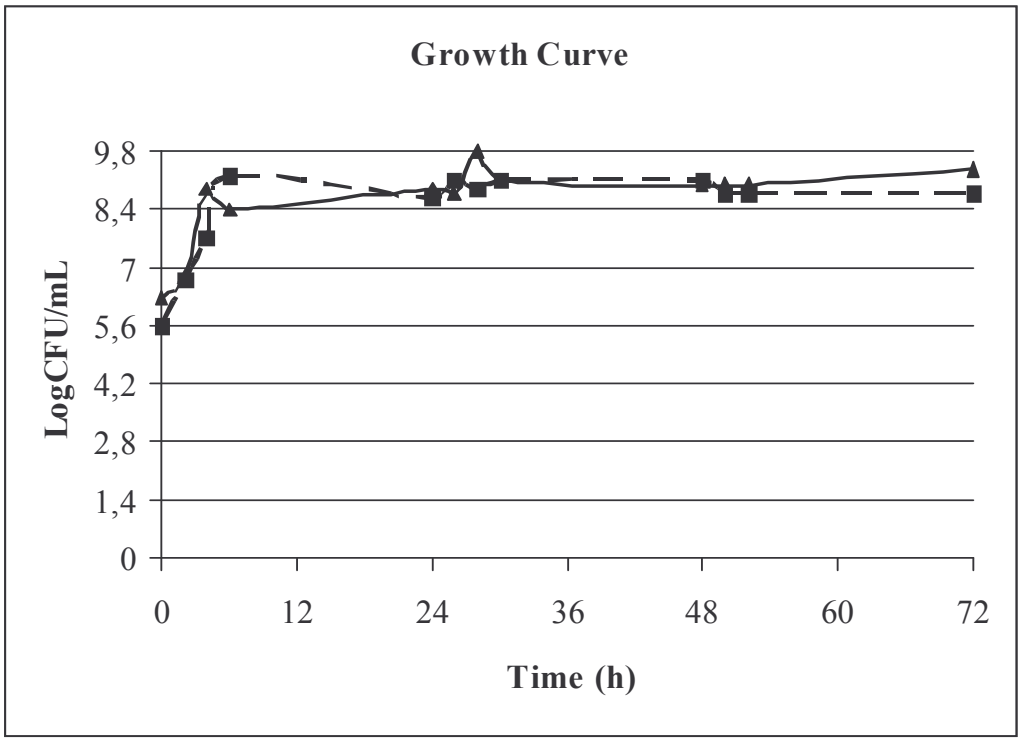


Figure 3.2. Growth curves for *Bti* HD500 (straight line) and *Bti* ONR60 (broken line) when cultivated in mYSM having the C:N ratio of 20:1.

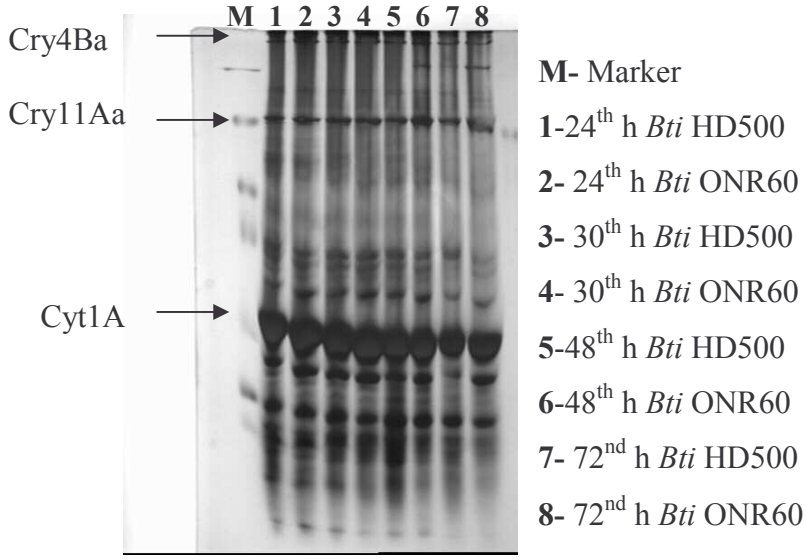


Figure 3.3. Effect of incubation time on Cry4Ba, Cry11Aa and Cyt1A delta-endotoxin production

The thicknesses of the protein bands other than Cry4Ba, corresponding to toxin proteins, were almost the same in the samples obtained from both the strains grown for 48 h. When incubation period was prolonged, however, Cry4Ba protein was diffused in both the strains. Therefore, among different incubation periods compared, 48 h seemed to be the most appropriate one considering the high levels of all major crystal toxins.

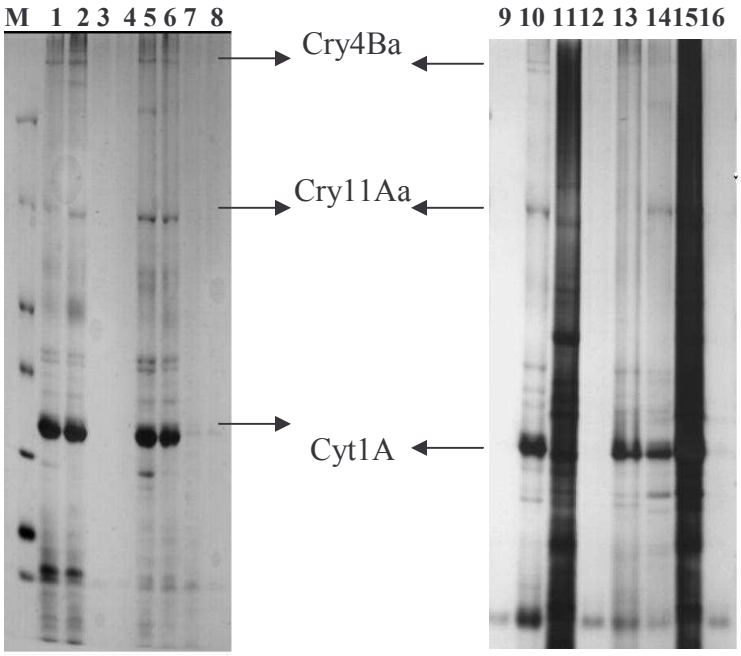
In the further experiments, two strains, grown in two different carbon sources (dextrin and sucrose), were compared for their toxin production capacities. The sugars in question were earlier found to be the most effective in toxin protein production (Özkan *et al*., 1997). The growth media contained different amounts of dextrin and sucrose to yield C:N ratios of 20:1 and 30:1 respectively. For both the

strains, the toxin protein bands (Figures 3.4) and wet weights of cells (Table 3.2) were measured at 24th and 48th hours of incubation in four different media with C:N ratios of with both sucrose and dextrin. Although *Bti* HD500 grew better within 24h in the medium containing dextrin as compared to that of sucrose, no toxin protein bands were apparent. Similarly, with *Bti* ONR60 the results were much the same since none of the toxin protein bands were seen when the carbon source was dextrin. These findings seemed to be contradictory to those of Özkan *et al.* (1997) who showed dextrin to be stimulatory for the biosynthesis of both Cry4Ba and Cry11Aa. However, the amount of dextrin used in the present study was 17 to 26 times higher in our experiments due to the prearranged C:N ratios. As seen in Figure 3.4, *Bti* HD500 showed no notable difference between 20:1 and 30:1 C:N ratios in terms of its crystal protein production. Whereas with ONR60, 20:1 ratio proved to be better that of 30:1 ratio in sucrose, as the latter yielded no toxin bands after 24th and 48th h of incubation. It should be noted that the amount of both the proteins (Cry4Ba and Cry11Aa) were low after the incubation period when the C:N ratio was 20:1.

Table 3.2. Effect of carbon source, C:N ratio and incubation time on cell yields (wet weight) of *Bti* HD500 and *Bti* ONR60.

Carbon source	C:N ratio	Wet weight of cells (g/mL)			
		Bti HD500		*Bti* ONR60	
		24th h	48th h	24th h	48th h
sucrose	20:1	0.01	0.01	0.04	0.02
	30:1	0.02	0.01	0.02	0.04
dextrin	20:1	0.02	0.01	0.02	0.04
	30:1	0.04	0.02	0.04	0.04

In view of the results shown in Figure 3.4, sucrose turned out to be the choice of carbon source, 48 h being the most appropriate time of incubation for further experiments.



M- Marker
1-24th h, 20:1 sucrose, *Bti* HD500
2- 24th h, 30:1 sucrose, *Bti* HD500
3- 24th h, 20:1 dextrin, *Bti* HD500
4- 24th h, 30:1 dextrin, *Bti* HD500
5-48th h, 20:1 sucrose, *Bti* HD500
6-48th h, 30:1 sucrose, *Bti* HD500
7- 48th h, 20:1 dextrin, *Bti* HD500
8- 48th h, 30:1 dextrin, *Bti* HD500
9- 24th h, 30:1 sucrose, *Bti* ONR60
10- 24th h, 20:1 sucrose, *Bti* ONR60
11- 24th h, 20:1 dextrin, *Bti* ONR60
12- 24th h, 30:1 dextrin, *Bti* ONR60
13-48th h, 30:1 sucrose, *Bti* ONR60
14-48th h, 20:1 sucrose, *Bti* ONR60
15- 48th h, 20:1 dextrin, *Bti* ONR60
16- 48th h, 30:1 dextrin, *Bti* ONR60

Figure 3.4. Effect of the carbon source, C:N ratio and incubation time on production of crystal proteins by *Bti* HD500 and *Bti* ONR60.

3.2. Response Surface Optimization

According to the principles of response surface methodology (RSM), it is inevitable to identify the important factors that can affect the product yield drastically. The method thus aims at studying such factors collectively under different sets of conditions that influence the response (Cornell, 1990).

In an earlier study of Özkan *et al*. (2003), the important factors effective in Cry4Ba and Cry11Aa protoxin production by *Bti* HD500 were reported as the temperature, Mn and K_2HPO_4 concentrations. According to the results of that study, the highest protoxin yields of Cry4Ba and Cry11Aa were obtained at 50-100 mM K_2HPO_4. The beneficial effects of Mn for both of the crystal proteins were only seen at 10^{-6} M. Also the highest concentrations of Cry4Ba were observed at 25°C, while the optimum temperature was 30°C for Cry11Aa. The present study revealed that C:N ratio was also an important factor which was set as 20:1 for HD500 by using sucrose as the carbon source. Taking all that into consideration, Cry4Ba and Cry11Aa yields were selected to be the responses for the method to be employed and [Mn], temperature, C:N ratios and [K^+] were selected to be the major factors with possible influence on responses. Prededermined optimum levels of these variables determined the boundaries of experimental design as shown in (Table 3.3).

Table 3.3. Experimental boundaries employed in this study.

Range and levels / Variables	-2	-1	0	1	2
X_1: log[Mn]	-8	-7	-6	-5	-4
X_2: T(°C)	25	27,5	30	32,5	35
X_3: C:N	10	15	20	25	30
X_4: K_2HPO_4 (mM)	0	25	50	75	100

According to these experimental boundaries, an experimental design was made by the use of the Design Expert 6.0 software as given at Table 3.4. This table is composed of 30 experiments, 24 sets of different experiments within the given 30

were the combinations of the coded values of the experimental variables and the remaining 6 were the central points. The use of Mn concentration as log [Mn] was due to our intent of correct numerical placement of the values on the number line with equal intervals. Running of 6 replicates at the central points was important to determine the curvature and to compensate the lack of fit values which indicate the significance of the model (Design Expert 6.0).

Table 3.4. Experimental design given with the coded values of the variables to be tested.

Experiment	log[Mn]	T (°C)	C:N	K_2HPO_4 (mM)
1	-1	-1	-1	-1
2	1	-1	-1	-1
3	-1	1	-1	-1
4	1	1	-1	-1
5	-1	-1	1	-1
6	1	-1	1	-1
7	-1	1	1	-1
8	1	1	1	-1
9	-1	-1	-1	1
10	1	-1	-1	1
11	-1	1	-1	1
12	1	1	-1	1
13	-1	-1	1	1
14	1	-1	1	1
15	-1	1	1	1
16	1	1	1	1
17	-2	0	0	0
18	2	0	0	0
19	0	-2	0	0
20	0	2	0	0
21	0	0	-2	0
22	0	0	2	0
23	0	0	0	-2
24	0	0	0	2
25	0	0	0	0
26	0	0	0	0
27	0	0	0	0
28	0	0	0	0
29	0	0	0	0
30	0	0	0	0

After these experiments have been performed, the Cry4Ba and Cry11Aa yields were determined for each set of experiments which were tabulated in Table 3.5.

Table 3.5. Cry4Ba and Cry11Aa yields of each experimental run

Experiment	Cry4Ba (mg/mL)	Cry11Aa (mg/mL)
1	0,0065	0,0018
2	0,0267	0,0589
3	0,0084	0,0022
4	0,0664	0,0486
5	0,0135	0,0312
6	0,0094	0,0064
7	0,0354	0,0336
8	0,0150	0,0100
9	0,0359	0,0689
10	0,0434	0,0324
11	0,0053	0,0046
12	0,0115	0,0114
13	0,0008	0,0193
14	0,0179	0,0370
15	0,0042	0,0038
16	0,0093	0,0230
17	0,0095	0,0169
18	0,0063	0,0000
19	0,0041	0,0180
20	0,0082	0,0113
21	0,0112	0,0167
22	0,0722	0,0235
23	0,0418	0,0297
24	0,0104	0,0283
25	0,0088	0,0094
26	0,0223	0,0016
27	0,0088	0,0055
28	0,0076	0,0043
29	0,0017	0,0053
30	0,0036	0,0156

For the analysis of the results listed in Table 3.5, the cubic response surface model was used. The application of the RSM yielded the following regression equations and 2. The equations reflect the empirical relationships between Cry4Ba and Cry11Aa and the test variables in coded units, respectively.

$$y_1 = 8.802\ x10^{-3} + 7.726\ x10^{-3}x_1 - 2.283\ x10^{-4}x_2 - 0.013x_3 - 1{,}824\ x10^{-3}x_4 - 4.143\ x10^{-4}\ x_1^2 - 8.430\ x10^{-4}x_2^2 + 8.039\ x10^{-3}x_3^2 + 4.138\ x10^{-3}x_4^2 + 5.144\ x10^{-4}x_1x_2 - 5.878\ x10^{-3}x_1x_3 - 1.106x10^{-3}x_1x_4 + 2.702\ x10^{-3}x_2x_3 - 8.561\ x10^{-3}x_2x_4 - 1.831\ x10^{-3}x_3x_4 - 2.129\ x10^{-3}x_1^3 + 3.127\ x10^{-4}x_2^3 + 7.141\ x10^{-3}x_3^3 - 1.506\ x10^{-3}x_4^3 - 4.063\ x10^{-3}x_1x_2x_3 - 2.178\ x10^{-3}x_1x_2x_4 + 6.954\ x10^{-3}x_1x_3x_4 + 4.472\ x10^{-3}x_2x_3x_4 \quad \text{(Eq. 3.1)}$$

$$y_2 = 6.935\ x10^{-3} - 6.602\ x10^{-3}x_1 - 9.327\ x10^{-3}x_2 - 0.013\ x10^{-3}x_3 + 7.367\ x10^{-4}x_4 + 1.456\ x10^{-3}x_1^2 + 3.011\ x10^{-3}x_2^2 + 4.366\ x10^{-3}x_3^2 + 6.606\ x10^{-3}x_4^2 - 2.2\ x10^{-3}x_1x_2 + 5.33\ x10^{-3}x_1x_3 + 2.998x10^{-3}x_1x_4 + 4.489\ x10^{-3}x_2x_3 - 6.936\ x10^{-3}x_2x_4 - 2.488\ x10^{-4}x_3x_4 + 2.704\ x10^{-3}x_1^3 + 1.91\ x10^{-3}x_2^3 + 1.911\ x10^{-3}x_3^3 - 2.704\ x10^{-4}x_4^3 + 1.862\ x10^{-3}x_1x_2x_3 - 3.405\ x10^{-3}x_1x_2x_4 + 0.014x_1x_3x_4 + 2.489\ x10^{-3}x_2x_3x_4 \quad \text{(Eq. 3.2)}$$

The R^2, adjusted R^2, F- value and Prob>F values were tabulated in Table 3.6 for both Cry4Ba and Cry11Aa.

Table 3.6. The statistical results of the cubic response surface models for Cry4Ba and Cry11Aa yields.

Type of Toxin	**Toxin Yields (mg/mL)**			
	R^2	**Adjusted R^2**	**F-value**	**Prob>F**
Cry4Ba	0.960	0.836	7.69	0.0050
Cry 11Aa	0.940	0.752	4.99	0.0180

The Prob>F less than 0.05 indicates the significancy of the model for both Cry4Ba and Cry11Aa having 0.0050 and 0.0180 Prob>F values, respectively. Also the high values of R^2, 0.960 for Cry4Ba and 0.940 for Cry11Aa, respectively, indicated that the fitness of the test was high for each of the crystal proteins. Moreover, the high values of adjusted R^2 values for Cry4Ba and Cry11Aa, being 0.836 and 0.752 respectively, indicated the significance of the model one more time for both of the crystal proteins Cry4Ba and Cry11Aa. The response surfaces obtained according to this analysis for Cry4Ba and Cry 11Aa are shown in Figures 3.5.a-f and 3.6.a-f, respectively. At all response surfaces, while the effects of two components to each other were being inspected, the remaining two variables were held constant at zero level. The significance of each coefficient for Cry4Ba and Cry11Aa was determined by F-value and Prob>F values which are listed in Table 3.7 and Table 3.8. The values of Prob>F less than 0.050 indicate that the model terms are significant. Response surface plots provide a method to predict the toxin protein yields for different values of the test variables and the contours of the plots help in identification of the type of interactions between test variables. The response surfaces having circular contour plots indicate that the interaction between the corresponding variables is negligible. An elliptical or saddle nature of the contour plots indicates the significancy of the interactions between the corresponding variables. In the case of saddle contour plots, the optimum values are obtained at the point of intersection of lines which are formed by joining the locus (Murthy *et al.*, 2000).

The response surface plot showing the mutual effect of temperature and log[Mn] on production of Cry4Ba (Fig. 3.5.a) indicates that the best protein yield was obtained when the log[Mn] was between -5.5 and -4.5, if the temperature was above 27°C . When the next Figure (Fig. 3.5.b) was examined, it was interpreted that the C:N ratio was itself enough to control the response, Cry4Ba concentration, without any effect of the incubation temperature. The highest yields of Cry4Ba could be obtained if both of the variables were kept at the highest points, 35°C for temperature and 30:1 for the C:N ratio. If the relationship between temperature and [K_2HPO_4] (Fig. 3.5.c) was interpreted, it can be seen that the Cry4Ba yields could be very high at 35°C in a medium having no K_2HPO_4. It thus appeared that the cells have effectively used up at this temperature the potassium provided by yeast extract and phosphate provided by $(NH_4)_2HPO_4$ and compensated the absence of K_2HPO_4. The interaction between [K_2HPO_4] and temperature was one of the important factors since at any of the concentrations of K_2HPO_4 below 50 mM, the response could be obtained at higher extents at a very broad temperature range between 26°C to 35°C. But, when the concentration of K_2HPO_4 was above 50 mM, the temperature alterations could not be tolerated and the yield decreased at the temperatures above 29°C. At the response surface showing the relationship between [K_2HPO_4] and C:N ratio, the highest Cry4Ba concentrations could be obtained if the C:N ratio was 30:1 and again there was no K_2HPO_4. However, if the contour was considered, the results also indicated that C:N ratio was more important than [K_2HPO_4]. According to our experimental

results, the highest yields of Cry4Ba toxin could be obtained when K_2HPO_4 concentration was higher than 26 mM while the C:N ratio was between 18:1 and 25:1. Likewise, Fig. 3.5.e showing that the response surface of [K_2HPO_4] and log[Mn] affects each other also indicated that the highest value of Cry4Ba could be obtained when log[Mn] was -6 and [K_2HPO_4] was zero. Besides, when the relationships of these two components were investigated, it could be seen that although they could not tolerate any alterations of the other when one was kept constant, there was a best [K_2HPO_4] value for each log[Mn] value in order to obtain the highest Cry4Ba yield. Lastly, according to the Figure 3.5.f, the response surface showing the relationship between C:N ratio and log[Mn], the highest Cry4Ba concentrations could be obtained if the C:N ratio was 30:1 and log[Mn] was -8. Their relationship was mostly dominated by C:N ratio, because although there was a corresponding ratio of C:N to every single value of log[Mn] yielding high Cry4Ba levels, the same condition was not applicable for the log[Mn] values corresponding to every C:N ratio to obtain high titers of this crystal protein. That is why the C:N ratio must be arranged before optimum Mn concentration is determined. When the response surfaces for Cry4Ba were examined again according to the perspectives of the above explanations, it could be easily interpreted that the most effective variable was C:N ratio (Prob>F= 0.0014) for toxin production (Fig.3.5.b, 3.5.d and 3.5.f). The highest values of Cry4Ba could be obtained at very narrow ranges of C:N ratios. Another important interaction that drastically affected Cry4Ba production was found between

temperature and [K_2HPO_4] (Prob>F= 0.0024) (Fig.3.5.c). For K_2HPO_4 concentrations above 50 mM and at temperatures below 30°C, the highest Cry4Ba yields could be obtained with a changing temperature when [K_2HPO_4] was kept almost constant. On the other hand, above 30°C, the temperature must be kept constant if the [K_2HPO_4] is to be changed to a higher concentration. The third important interaction which was observed between log[Mn], C:N ratio and [K_2HPO_4] (Fig.3.5.e and 3.5.f) triplet and it had a Prob>F value of 0.0071. The interaction between temperature, C:N ratio and K_2HPO_4 concentration was also one of the significant interactions with a Prob>F value of 0.0463.

Table 3.7. F-values and Prob>F values for Cry4Ba model terms.

Model Terms	F-value	Prob>F value
x_1	8.72	0.0213
x_2	$7.615x10^{-3}$	0.9329
x_3	25.84	0.0014
x_4	0.49	0.5082
x_1^2	0.086	0.7779
x_2^2	0.36	0.5696
x_3^2	32.37	0.0007
x_4^2	8.58	0.0221
x_1x_2	0.077	0.7890
x_1x_3	10.09	0.0156
x_1x_4	0.36	0.5690
x_2x_3	2.13	0.1876
x_2x_4	21.41	0.0024
x_3x_4	0.98	0.3554
x_1^3	3.97	0.0865
x_2^3	0.086	0.7782
x_3^3	44.69	0.0003
x_4^3	1.99	0.2013
$x_1x_2x_3$	4.82	0.0641
$x_1x_3x_4$	1.39	0.2776
$x_1x_3x_4$	14.13	0.0071
$x_2x_3x_4$	5.84	0.0463

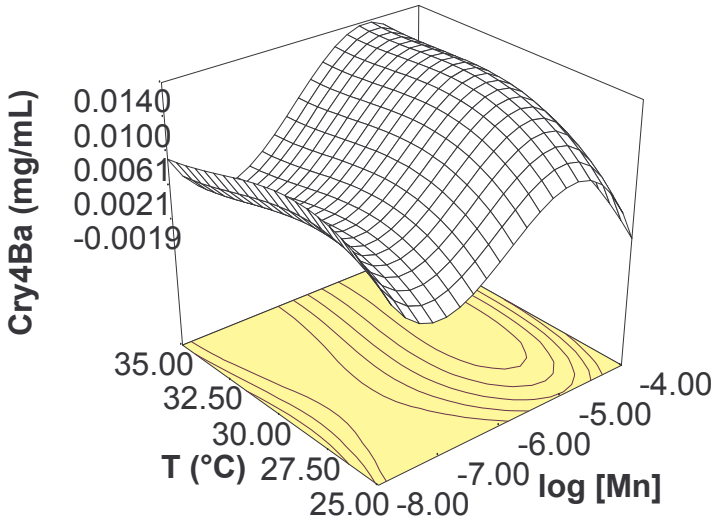


Figure 3.5.a. Response surface plot showing the mutual effect of temperature and log[Mn] on the production of Cry4Ba when the other variables are held at zero level.

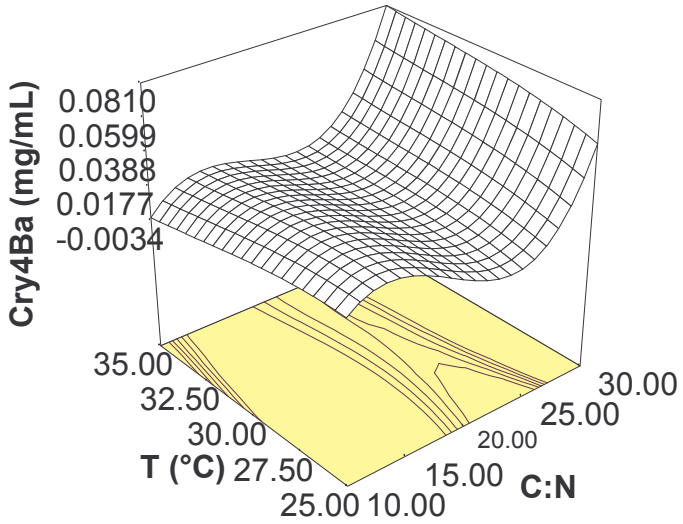


Figure 3.5.b. Response surface plot showing the mutual effect of temperature and C:N ratio on the production of Cry4Ba when the other variables are held at zero level.

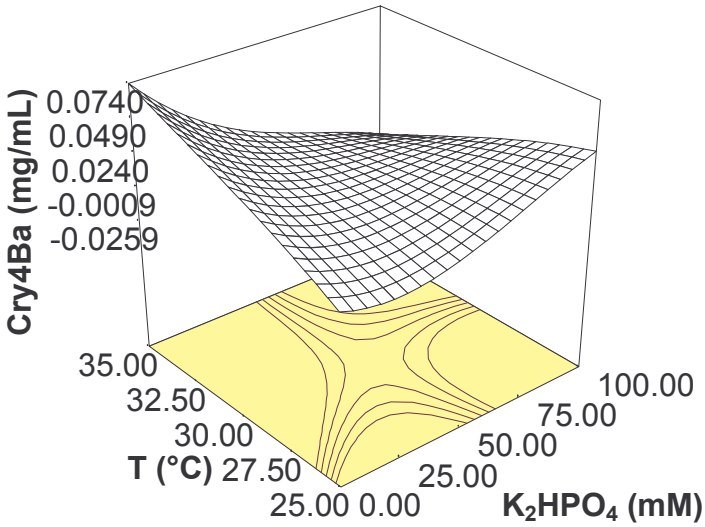


Figure 3.5.c. Response surface plot showing the mutual effect of temperature and K_2HPO_4 concentration on the production of Cry4Ba when the other variables are held at zero level.

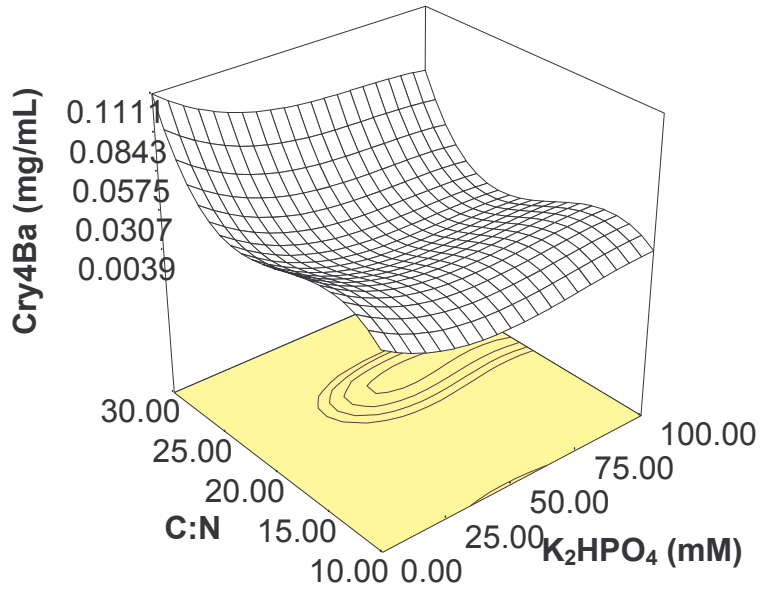


Figure 3.5.d. Response surface plot showing the mutual effect of C:N ratio and K_2HPO_4 concentration on the production of Cry4Ba when the other variables are held at zero level.

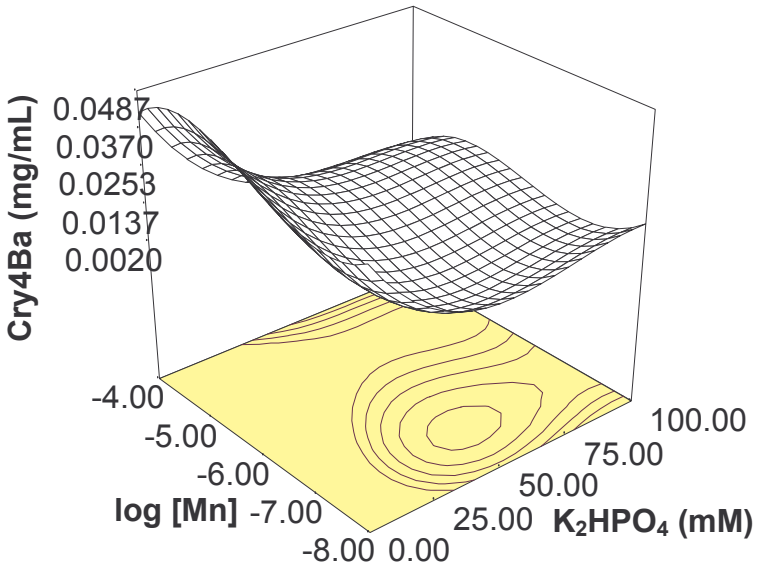


Figure 3.5.e. Response surface plot showing the mutual effect of log[Mn] and K_2HPO_4 concentration on the production of Cry4Ba when the other variables are held at zero level.

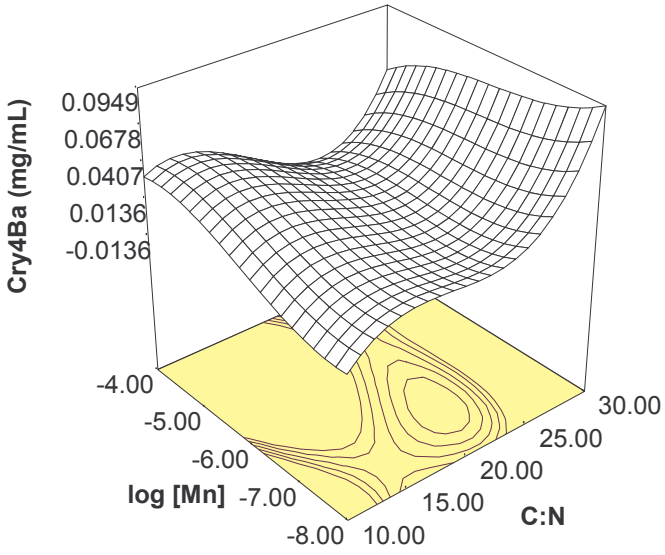


Figure 3.5.f. Response surface plot showing the mutual effect of C:N ratio and log[Mn] on the production of Cry4Ba when the other variables are held at zero level.

The response surfaces were also plotted for Cry11Aa. Fig. 3.6.a shows the interaction between incubation temperature and log[Mn]. According to this response surface, the highest Cry11Aa titers could be obtained at the edges of the scale (when temperature was 25°C and log[Mn] was -8, and when the temperature was 35°C and log[Mn] was -4). When their effects on the response were considered, there was a corresponding log[Mn] between 30°C and 25°C. This explains why their relationship was not very important for the yields of Cry11Aa response. When the second response surface, temperature:C:N effect was investigated for Cry11Aa (Figure 3.6.b), it was observed that the highest Cry11Aa yields were obtained at the edges of the surface, when temperature was 35°C and C:N ratio was 30:1 and also when temperature was 25°C and C:N ratio was 10:1. K_2HPO_4 concentration and temperature relationship (Figure 3.6.c) could yield the highest Cry11Aa yields at one point, that is, when the temperature was 25°C and K_2HPO_4 concentration was 100 mM. When this relationship was investigated, it could be seen that small changes could not be tolerated although there were two [K_2HPO_4] values corresponding to a given temperature. Therefore, the interaction of these components was found to be relatively more important than the previous two response surfaces. Figure 3.6.d shows the interaction between C:N ratio and log[Mn]. When this response surface was interpreted, it was obviously seen from the contour plots that at a broad range of changing C:N ratio, very little alteration was tolerated for log[Mn] to keep the titers of Cry11Aa at the highest level or the C:N ratio range became narrower while the range of log[Mn] increased. This

interaction was significant with a Prob>F value of 0.0437. According to the response surface plotted by these components, the highest Cry11Aa yields could be obtained when the C:N ratio was 30:1 and log[Mn] was -8. Figure 3.6.e shows that the highest Cry11Aa titers could be obtained if [K_2HPO_4] was 100 mM and log[Mn] was -8. The last response surface for Cry11Aa is shown in Figure 3.6.f. According to the contour plot of the response surface, it could be concluded that these components, K_2HPO_4 concentration and C:N ratio, had no effect on each other and their relationship was the least important among all response surfaces.

The response surfaces for Cry11Aa revealed that the interaction of [K_2HPO_4] with the temperature was very important (Prob>F= 0.0151) (Fig 3.6.c). Small alterations in both [K_2HPO_4] and temperature interfered with high level production of Cry11Aa. Like Cry4Ba, Cry11Aa yield seemed to be affected by the relationship between log[Mn], C:N and [K_2HPO_4] (Prob>F= 0.0004) (Fig 3.6.d and 3.6.e). Log[Mn] and C:N ratio did also constitute an important interaction with the Prob>F= 0.0437, but their interaction is less important as compared to the above mentioned ones.

When the general profile was considered, it was seen that the interactions between the test values more significantly affect the biosynthesis of Cry4Ba than that of Cry11Aa biosynthesis, as can be deduced from the Prob>F values for Cry4Ba and Cry11Aa (Table 3.7 and 3.8).

Table 3.8. F-values and Prob>F values for Cry11Aa model terms

Model Terms	F-value	Prob>F value
x_1	4.63	0.0685
x_2	9.23	0.0189
x_3	3.74	0.0944
x_4	0.058	0.8172
x_1^2	0.77	0.4087
x_2^2	3.30	0.1121
x_3^2	6.94	0.0337
x_4^2	15.89	0.0053
x_1x_2	1.03	0.3445
x_1x_3	6.03	0.0437
x_1x_4	1.91	0.2097
x_2x_3	4.28	0.0774
x_2x_4	10.21	0.0151
x_3x_4	0.013	0.9120
x_1^3	4.66	0.0678
x_2^3	2.32	0.1712
x_3^3	2.33	0.1710
x_4^3	0.047	0.8353
$x_1x_2x_3$	0.74	0.4192
$x_1x_3x_4$	2.46	0.1607
$x_1x_3x_4$	39.59	0.0004
$x_2x_3x_4$	1.32	0.2892

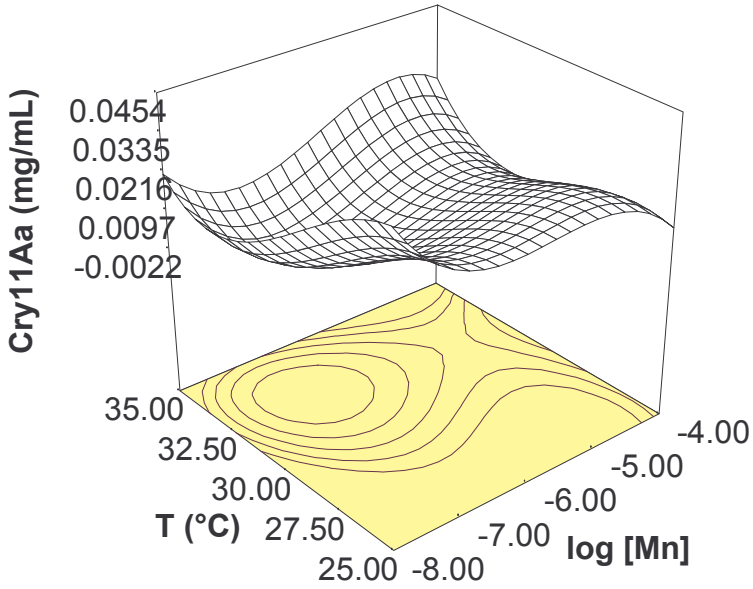


Figure 3.6.a. Response surface plot showing the mutual effect of temperature and log[Mn] on the production of Cry11Aa when the other variables are held at zero level.

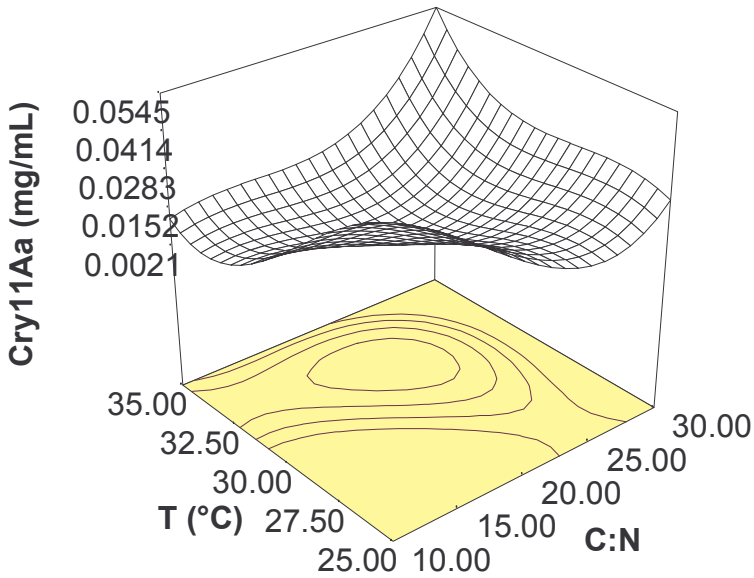


Figure 3.6.b. Response surface plot showing the mutual effect of temperature and C:N ratio on the production of Cry11Aa when the other variables are held at zero level.

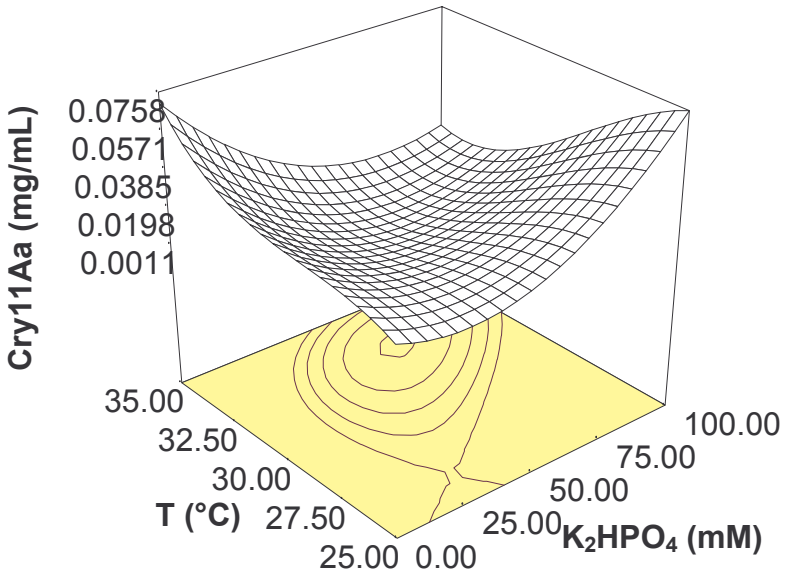


Figure 3.6.c. Response surface plot showing the mutual effect of temperature and K_2HPO_4 concentration on the production of Cry11Aa when the other variables are held at zero level.

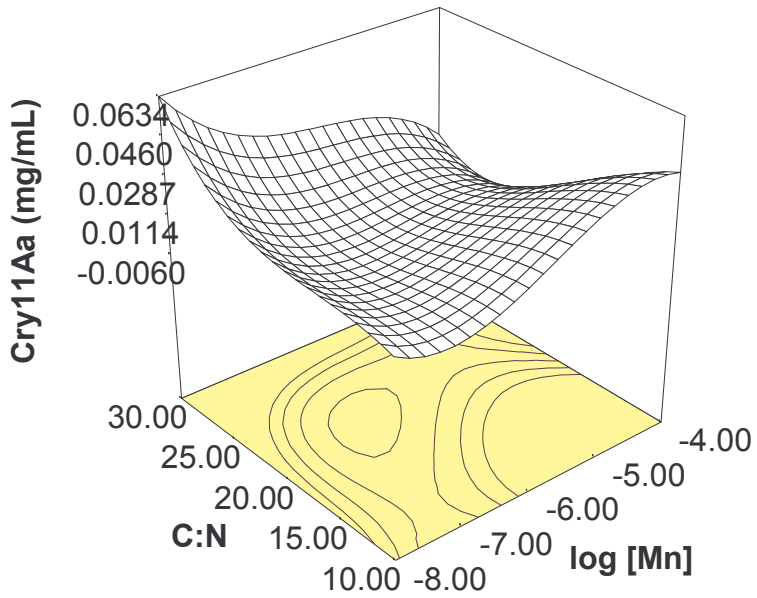


Figure 3.6.d. Response surface plot showing the mutual effect of C:N ratio and log[Mn] on the production of Cry11Aa when the other variables are held at zero level.

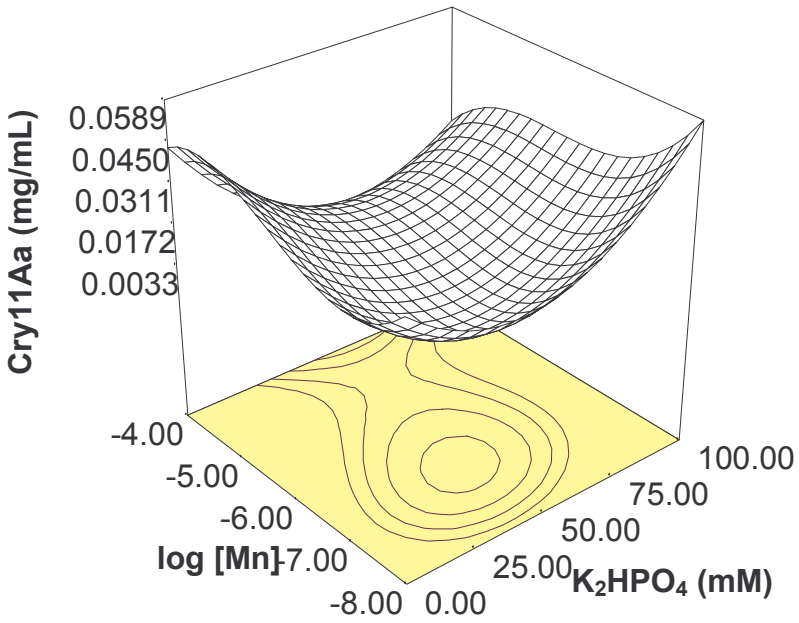


Figure 3.6.e. Response surface plot showing the mutual effect of log[Mn] and K_2HPO_4 concentration on the production of Cry11Aa when the other variables are held at zero level.

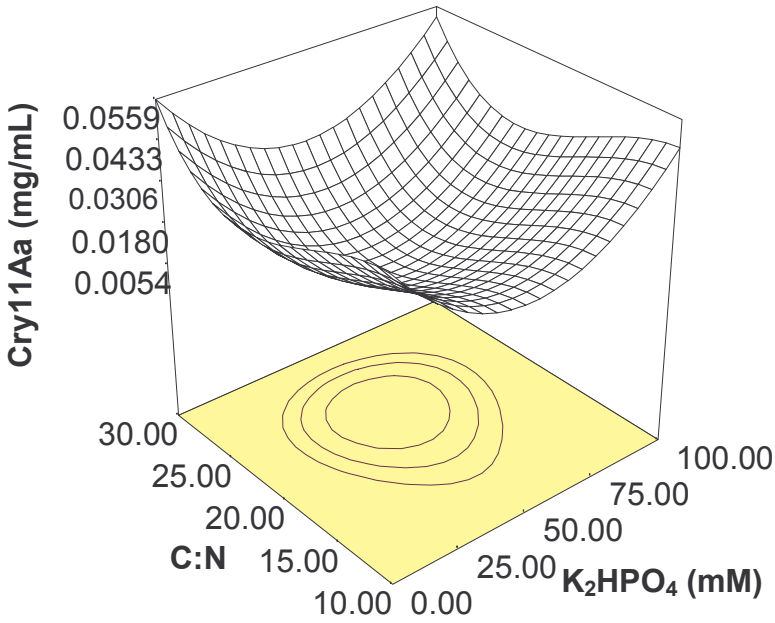


Figure 3.6.f. Response surface plot showing the mutual effect of C:N ratio and K_2HPO_4 concentration on the production of Cry11Aa when the other variables are held at zero level.

In a recent study conducted by Wirth *et al.* (2003), the *cyt1A* and *cry11Aa* genes were deleted separately and with the mutants having the *cyt1A* deletion, the investigators observed that the mosquito strains developed resistance. This study indicated the importance of Cyt1A protein for the mosquito control. The response surfaces for Cyt1A have not been analysed in the present study since it was previously shown that the yields of Cyt1A were not much influenced by the changes in medium composition and temperature (Özkan *et al*, 2003). In the present study, the concentrations of Cyt1A ranged between 0.4 to 0.6 mg/mL in all the experiments, supporting the findings of Özkan *et al.* (2003).

The response surfaces showed the optimal levels of variables for Cry4Ba and Cry11Aa proteins, individually. However, it was important to find out the conditions under which the yields of both of the toxin proteins were optimal. The conditions were as follows in the optimum solution selected from DesignExpert 6.0 software: Temperature, 28.3°C; [Mn], $3.3x10^{-7}$M; C:N ratio, 22.2 and [K_2HPO_4], 66.1 mM. The corresponding experiment was carried out in five replicates and the average value was obtained. The toxin protein yields were 0.0289 mg/mL for Cry4Ba and 0.0692 mg/mL for Cry11Aa while the predicted values were 0.028 mg/mL for Cry4Ba and 0.029 mg/mL for Cry11Aa. Thus, the comparison of the experimental and the predicted values indicated that the use of response surface model for Cry4Ba production (Eq. 3.1) is more reliable than that for Cry11Aa production (Eq. 3.2). It is to be noted that a *cry11A* deletion mutant

of *Bt israelensis* was equally or more toxic than native *Bt israelensis*, as was verified very recently (Wirth *et al*., 2003), thus the optimization of Cry4Ba yields by RSM seemed to be more meaningful in our case.

When the yields obtained in this study were compared to the Cry4Ba and Cry11Aa yields reported by Özkan *et al.* (2003), the yields were found to be higher in the present study. This can be due to the C:N ratio rearrangement in the medium that we made. In the previous medium (mYSM), the sugar concentration (sucrose) was rather low, 1 gL^{-1} (to give 1:1 C:N ratio), which was increased in the present study to 19.3 gL^{-1} (20:1). At the optimum level determined by using RSM, this amounted to 21.4 gL^{-1} (22.2:1) which might have allowed the bacteria to utilize more sugar without causing drastic pH changes as owing to a balanced C:N ratio. Vidyarthi *et al.* (2002) reported that a C:N ratio ranging between 7.9-9.9 was good for *Bt kurstaki* grown in wastewater sludge. Farrera *et al.* (1998), on the other hand, reported a C:N ratio of 7:1 to be the best for the same subspecies.

Özkan *et al.* (2003) was reported about the significance of using high concentrations of K_2HPO_4 (50 – 100 mM) in order to obtain high titers of Cry4Ba and Cry11Aa. In the present study, [K_2HPO_4] was found to be optimal at 66.1 mM which falls in the suggested range. When the generalization that 0.3 – 300 mM inorganic phosphate supports good growth while the concentrations higher than 10 mM suppresses secondary metabolism is remembered (Demain, 1982),

this concentration of K_2HPO_4 can be found to be out of the borders. Nonetheless, Bhatnagar (1998) also reported the beneficial effect of inorganic phosphate addition on Cry4A protoxin synthesis in *Bti*.

3.3. *Bti* HD500 Release Experiments By Using Granulated Formulations

For both mosquito and blackfly control, the culture preparations composed of spores and crystal proteins must be converted to an easily applicable and persistant forms for long-lasting bioactivity. All formulation strategies are made to increase the field effectiveness of the product against mosquitoes and blackflies (Guillet *et al*., 1985a; Lacey and Heitzman, 1985). The best known *Bt* formulations are called wettable powders or granules which are easily prepared by using chemical techniques (Dulmage, 1970). It was shown that the partical size was directly important for the toxicity of the formulation since increase in size of the particles increases toxicity as well (Guillet and Escaffre, 1979; Molloy *et al*., 1984; Guillet *et al*., 1985b). On the other hand, although such formulations have larger particle sizes than liquid ones, the latter is regarded to be more practical at field trials (Guillet *et al*., 1982; Lacey and Undeen, 1984; Molloy *et al*., 1984). Other important factors are the dose and the duration of the application. The higher the dose and duration, the better the effect, as expected (Molloy *et al*., 1990). As stated before, either liquid, powder or granule, it is important to keep

the formulations for longer periods of time at the applied area. Another important requirement for all preparations is to release their contents slowly when they contact with water.

In the context of our research and development project aiming at the manufacture of *Bti* product locally, *Bti* HD500 was grown at a large scale and spores plus crystal protein mixtures were collected (ORBA Biochemicals Co.). Different granulated formulations of this product were made by Prof. E. Bayramlı (The Chemistry Department of METU). The formulations were then tested for their anti-larvicidal activity at Hacettepe University by Dr. Öner Koçak as well as release properties in our laboratories. Experimentally, 0.5 g of the formulation was placed in a sterile container to which 10 mL of sterile dH_2O was added. At time intervals, the suspension in the container was mixed throughly, a 100 μL aliquot was taken, serially diluted and subjected to viable count to determine the number of spores released in terms of CFU/mL (Fig.3.7). As seen, a sharp increase in counts was observed, indicating that whole content of the granules had been released within 6 h. From this particular point onwards, the viable counts did not change for about 9 days, indicating that a steady-state was reached (Figure 3. 7). Our expectation was however, to observe a gradual increase in viable counts against time (an indication of continuous release of toxins and spores).

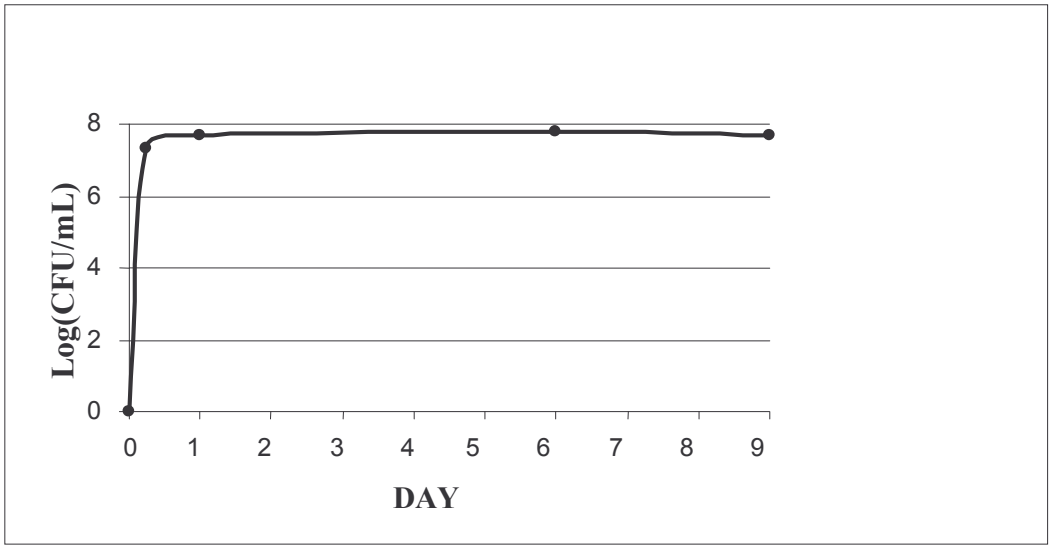


Figure 3.7. Viable counts for *Bti* HD500 from the granulated preparation.

On the basis of the findings of this particular experiment; the granular preparation proved to be inappropriate and a new formulation was thus developed. The release profile of this particular preparation was studied through a new approach; the formulation was placed in a sterile sac to be dipped into known volume of sterile water. The water in the container was changed every day. By this way, the net release per day could be determined (Figure 3.8). The results showed that the release continued during 15 days, although the amount of *Bti* HD500 spores released showed a slight and gradual decrease after the 2nd day. The presence of spores at zero time could be due to the presence of small particles that were able to pass from the sac into the water.

This experiment verified that our *Bti* product has been formulated properly to ensure sustained release in field applications for at least 2 weeks to control mosquitoes.

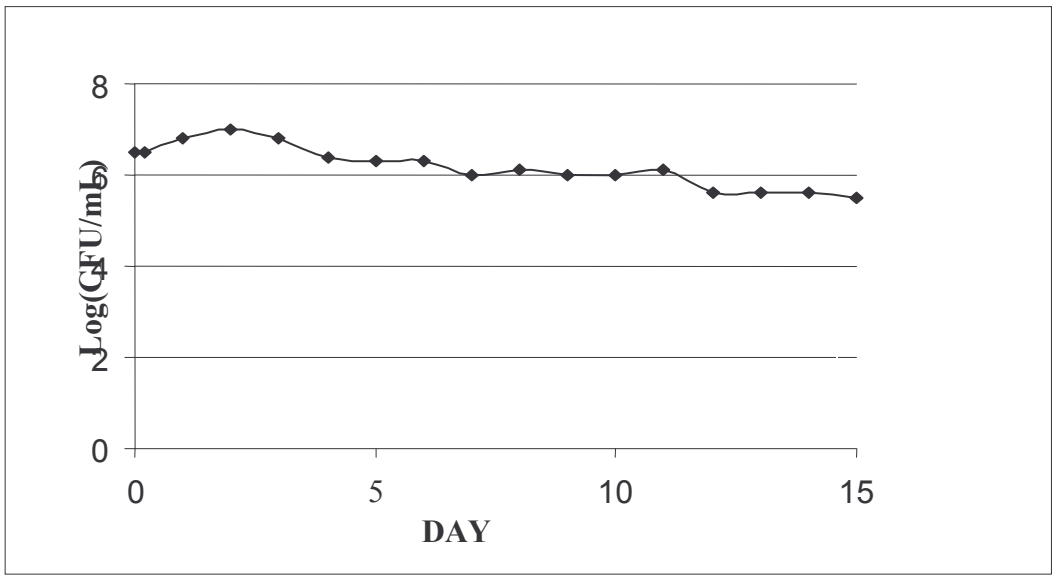


Figure 3.8. Viable counts for *Bti* HD500 from the improved granulated formulation by changing the water everyday.

CHAPTER 4

CONCLUSION

- For both the strains *Bti* HD500 and *Bti* ONR60, among different incubation periods compared, 48 h seemed to be most appropriate one considering the high levels of all major crystal toxins.
- The toxin protein production capabilities of both the strains with two different carbon sources, dextrin and sucrose, was compared. Sucrose was selected as a carbon source of choice.
- Since the production of crystal proteins was better in *Bti* HD500 as compared to of *Bti* ONR60, the former was selected for further studies.
- The C:N ratio of 20:1 yielded highest amount of Cry4Ba toxin and fairly good amount of Cry11Aa component.
- The application of the RSM yielded regression equations that reflect the empirical relationships between Cry4Ba and Cry11Aa and the test variables in coded units.
- The most effective variable was C:N ratio (Prob>F= 0.0014) for Cry4Ba toxin protein production.

- For Cry11Aa toxin protein production, relationship between log[Mn], C:N and [K_2HPO_4] (Prob>F= 0.0004) was very important.
- The interactions between the test values were found to have more significant affect on the biosynthesis of Cry4Ba than that of Cry11Aa.
- The optimum solution under which the yields of both the toxin proteins were expected to be optimal should contain; [Mn], $3.3x10^{-7}$M; C:N ratio, 22.2 and [K_2HPO_4], 66.1 mM, the incubation temperature being 28.3°C.
- The comparison of the experimental and the predicted values indicated that the use of response surface model for Cry4Ba production is more reliable than that for Cry11Aa production.

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APPENDIX A

CULTURE MEDIA COMPONENTS

1. Modified Yousten's Synthetic Medium (Özkan *et al, 1997, 2003)*

Component	*Concentration* (g/L)
$CaCl_2$. $2H_2O$	0.08
K_2HPO_4	8.71
$MgSO_4$. $7H_2O$	0.04
$MnSO_4$. H_2O	0.169 mg
$(NH_4)_2HPO_4$	2
Yeast extract	2
Sucrose (C:N 4:1)	0.386
Sucrose (C:N 7:1)	0.676
Sucrose (C:N 10:1)	0.987
Sucrose (C:N 20:1)	19.3
Sucrose (C:N 30:1)	29
Dextrin (C:N 20:1)	17.4
Dextrin (C:N 30:1)	26.3

The pH was adjusted to 7.3 and the medium was sterilized at 121°C for 15 min.

2. Nutrient Agar Medium

Component	*Concentration* (g/L)
Nutrient broth	8
Agar	15

The pH was adjusted to 7.3 and the medium was sterilized at 121°C for 15 min.

APPENDIX B

BUFFERS AND REAGENTS

1. TE Buffer

10 mM Tris HCl

1 mM EDTA

pH 8

2. Stacking Buffer

6 g of Trizma base was dissolved in 80 mL distilled water, pH was adjusted to 6.8 with concentrated HCl then the volume was completed to 100 mL.

3. Separating Buffer

36.6 g Trizma base

48 mL of 1 N HCl.

Diluted to 100 mL with distilled water.

4. Running Buffer

30 g Trizma base

144 g Glycine

5 g SDS

Volume was made up to 5 L with distilled water.

5. Loading Buffer

Autoclaved glycerol	5	mL
10 % SDS	0.5	mL
β- mercaptoethanol	0.5	mL
Stacking buffer	2.5	mL
Sterile dH_2O	11.5	mL
Bromophenol blue	10	mg

6. Bradford reagent

100 mg Coomassie brilliant blue G was dissolved in 50 mL absolute ethyl alcohol and mixed for 6 hrs (or overnight) with light protection. 100 mL of 85 % ortho-phosphoric acid was added to this mixture and stirred for another 6 hrs (or overnight). The required volume from this stock was diluted to the desired volume with distilled water.

APPENDIX C

CHEMICALS AND THEIR SUPPLIERS

Acetic acid	Merck
Acrylamide	AppliChem
Agar- agar for microbiology	Merck
$AgNO_3$	Merck
Ammonium persulfate	AppliChem
Bromophenol blue	AppliChem
BSA	Sigma
$CaCl_2$. $2H_2O$	Merck
Dextrin (Type I)	Sigma
Ethanol	TEKEL
Formaldehyde	Merck
Glucose	Merck
Glycerol	Sigma
Glycine	Merck
HCl	Merck
K_2HPO_4	Merck
Lysozyme	Sigma
Mercaptoethanol	Merck

Methanol	Merck
$MgSO_4$	Ventron
$MnSO_4.\ 7H_2O$	Sigma
N, N'-Methylene-bis-acrylamide	Sigma
Na_2CO_3	Merck
$Na_2S_2O_3.\ 5H_2O$	Carlo Erba
NaCl	Carlo Erba
$(NH_4)_2HPO_4$	Merck
Nutrient broth	Pronadisa
Protein molecular weight markers	Fermentas
SDS	AppliChem
Sucrose	Merck
TEMED	Sigma
Tris (Trizma) Base	AppliChem
Tris HCl	AppliChem
Yeast extract	Difco