PRODUCTIVITY ANALYSES IN FERMENTATIONS WITH THREE DIFFERENT BIOLARVICIDES

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ABSTRACT

PRODUCTIVITY ANALYSES IN FERMENTATIONS WITH THREE DIFFERENT BIOLARVICIDES

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The development of insecticides resistance among many insect species and the ecological damage occasionally caused by the lack of specificity in the toxic effects of insecticides have provided the impetus to seek alternative methods of insect control. This observation led to the development of bioinsecticides based on the insecticidal action *Bacillus sphaericus* (*Bs*), *Bacillus turingiensis* (*Bt*).

The discovery of biolarvicidal actions of *Bacillus thuringiensis* and *Bacillus sphaericus* opened a new perspective for insect control.

In the first part of the study was initiated to determine a suitable fermentation medium formulation and optimal fermentation conditions for large scale, low cost production of *Bs. Bs 2362* was tested in whey and soy flour based media. These media was reformulized form of NYSM (Nutrient Broth Yeast Extract Sporulation Medium). Soy flour based medium, SYSM, gave the promising results in terms of cell yield, sporulation frequency and toxin production.

In the second part of the study, fermentation productivity anlaysis of a local isolate *Bacillus thuringiensis* subsp. k*urstaki* 81 was evaluated. In order to compare different C:N ratios (1:1, 2:1, 4:1, 8:1, 10:1 20:1 and 30:1) of YSM medium. *Btk* 81 were run for 72 h and cell growth, sporulation and toxin protein profile of *Btk* 81 were determined for each. When all the quantitative toxin data for both glucose and sucrose varying C:N ratios were compared, it was determined that the crystal protein concentrations had the highest value in sucrose based medium when C:N ratio was 10:1.

Regulation by C:N ratio of crystal protein biosynthesis was investigated for improving the production of this protein by our third candidate strain *Bacillus thuringiensis* subsp. *israelensis* ONR60. The experiments were performed by using TBL medium, at three different C:N ratios, 2:1, 4:1 and 8:1 respectively. In

view of the cell growth characteristics and bioassy results, TBL medium designed with 2:1 C:N ratio was chosen as the best for further steps. In addition, running time of the culture determined as 60 hours as was also determined in the previous experiment.

As the last step of this study, the pre-determined optimal conditions were applied to a 30L batch type fermentor for toxin production by using *Bacillus thuringiensis* subsp. *israelensis* ONR60. Unfortunately, the toxicity was not satisfactory, being much below the level of that expected as based on the results of the laboratory scale studies.

Key words: *Bacillus thuringiensis* subsp. *israelensis*, *Bacillus thuringiensis* subsp. *kurstaki*, *Bacillus sphaericus*, delta endotoxin, crystal protein, binary toxin, fermentation, carbon to nitrogen ratio, bioassay.

ÖZ

ÜÇ FARKLI BİYOLARAVASİDAL BAKTERİ SUŞUNDA FERMENTASYON VERİMLİLİĞİ ANALİZİ

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Birçok böcek türünün kullanılan insektisitlere karşı gösterdiği dirençlilik ve kullanılagelen insektisitlerin hedef profili dışındaki organizmalara zararlı etkileri mücadelede yeni amaçlara yönelimlere neden olmuştur. *Bacillus thuringiensis(Bt)*

ve *Bacillus sphaericus(Bs)* gibi bakterilerin insektisit özelliği gösteren aktivitilerinin bulunuşu.

Çalışmamızın ilk bölümünde büyük ölçekli ve düşük maliyetli üretim koşullara ön hazırlık olarak, fermentasyonda kullanılacak besiyerinin optimizasyonuna gidilmiştir. *Bs 2362* suşu, peynir altı suyu tozu ve soya unu temelli iki besiyerinde üretilmiştir. Kullanılan bu iki besiyeri NYSM easas alınarak yeniden tasarlanmıştır. Soya ununun karbon ve azot kaynağı olarak kullanıldığı SYSM hücre sayısı, sporlanma frekansı ve toksin üretimi açısından başarılı sonuçlar vermiştir.

Çalışmanın ikinci bölümünde local bir izolat olan *Bacillus thuringiensis* alt tür k*urstaki* 81'in verimlilik analizi yapılmıştır. YSM besiyeri 1:1, 2:1, 4:1, 8:1, 10:1 20:1 ve 30:1 C:N oranları glükoz ve sükroz kullanılarak yeniden dizayn edilmiş ve herbiri için 72 saatlik üretim sürecinde organizmanın büyüme, sporlanma ve protein üretim profilleri çıkarılmıştır.

Üçüncü suş olarak tercih edilen *Bacillus thuringiensis* subsp. *israelensis* ONR60 kristal protein üretimi, farklı C:N oranlarında tasarlanan TBL besiyerinde çalışılmış, bu amaçla 2:1, 4:1 ve 8:1 olamk üzere üç oran denenmiştir. Çalışmalar ve larva test sonuçları 2:1 C:N oranının sonraki basamaklar için tercih edilebilir olduğunu göstermiştir. En ideal üretim süreci 60 saat olarak belirlenmiştir.

Çalışmanın son basamağında halihazırda belirlenmiş olan optimal koşullar 30litrelik fermentöre uygulanmış, *Bacillus thuringiensis* alt tür. *israelensis* ONR60 suşunun larvasidal aktivitesi test edilmiştir. Sonuçlar beklenen değerlerin altında kalmıştır.

Anahtar Kelimeler: *Bacillus thuringiensis israelensis*, *Bacillus thuringiensis kurstaki, Bacillus sphaericus,* delta-endotoksin, kristal protein, ikili toksin.

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Bt	:	*Bacillus thuringiensis*
Bti	:	*Bacillus thuringiensis* subspecies *israelensis*
Btk	:	*Bacillus thuringiensis* subspecies *kurstaki*
Bs	:	*Bacillus sphaericus*
kDa	:	Kilodalton
MDa	:	Megadalton
YSM	:	Yousten's Synthetic Medium
mYSM	:	modified Yousten's Synthetic Medium
NYSM	:	Nutrient Broth Yeast Extract Synthetic Medium
SDS-PAGE	:	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

CHAPTER 1

INTRODUCTION

1.1 Microbial Insecticides

The competition for crops between humans and insects is as old as agriculture, but chemical warfare against insects has much shorter history. Farmers began to use chemical substances to control pests in the mid-1800s. Not suprisingly, the development of insecticides paralleled the development of chemistry. Early insecticides were in the main inorganic and organic arsenic compounds, organophosphates, carbamates, pyretroids and formamidines, many of which are in use today. The use of organic chemical pesticides has proven to be remarkly effective in aiding the development of modern agriculture and in limiting spread of a number of diseases that transmitted by insect vectors. Global sales of chemical insecticides have now reached about 5 billon dollars a year.

Nonethless, there are disadvantages of relying on exculisively chemical pesticides. The most impotant one is that the widespread use of a single chemical compounds confers a selective evolutionary advantage on the progeny of pests. Since they have acquired resistance to such chemicals. For example, housefly strains worldwide have developed resistance to virtually every insecticide used

against them. Another problem is that some pesticides affect nontarget species with disastrous results. Unexpected elimination of desirable predator insects has caused explosive multiplication of secondary pests. The last concern is the environmental persistence and toxicity of many pesticides and increased cost of developing new and safer ones. Totally, these disadvantages provide strong intention to find alternative approaches for pest control.

Like all living things, insects are susceptible to infection by bacteria, fungi, protozoa and viruses. Thus, these agents have narrow host ranges and do not cause random destructive actions on beneficial insects and they are not toxic to vertebrates. The discovery of the larvacidal action of some bacterial strains from genus B*acillus* was changed the picture. *Bacillus thuringiensis* has been used for pest control since 1920s and still accounts for over 90% of the biological insecticide market. Then another strong alternative, namely *Bacillus sphaericus* was next identified as a new biological insecticidal agent and Bs based products appeared in the market. Alternatively, insect viruses (baculoviruses) have achieved some modest commercial success as pest control agents. Still, further genetic manipulations of already defined agents or continuous isolation of new strains may well increase the utility of biological pest management strategies.

1.2. *Bacillus sphaericus* and Binary Toxins

The name *Bacillus sphaericus(Bs)* first applied by Neide in 1904 to an aerobic bacterium that formed terminally located, spherical spores. Kellen during routine surveillance of rock holes in California collected several moribund fourth instar larvae of *Culiseat incidens*. He isolated several bacteria, among which was a *Bs.* Kellen's strain was later designated as strain K. It was the first reported active *Bs* isolate (Kellen and Mayer, 1964). The isolation of strain SSII-1 (Singer 1973) renewed interest in *Bs.* In early 1975, starin 1593 was identified. It was the first fermentation and population stable strain. Wickremesinghe and Mendis (1980) isolated what they called MR-4 which has since been given the designation 2297. Strain 2362 was isolated by J. Weiser, (1984) and it has proven to be somewhat more toxic than 1593. Strains 2297, 1593 and 2362 remain the principal candidates of field interest.

The principal phenotypic traits used to place strains into *Bs* species are the presence of spherical spores, the inability to grow anaerobically, and negative reaction on variety of tests developed primarily for the calssification of the family *Enterobacteriaceae* (Yousten, 1984; Claus and Berekeley, 1986). In view of the fact that *Bs* is defined by the absence of positive traits, it's not suprising that *in vitro* DNA-DNA hybridization studies indicated that the species is highly heterogeneous and contains DNA homology groups deserving distinct species status (Krchy *et al.* 1980). Recently, broader taxonomic approaches involving an expanded nutritional screening and numerical analysis of the data

indicated that these strains constitute a number of distinct clusters, in aggreement with the result of DNA homolgy studies (Alexander and Priest, 1990). Since the clusters are not readily distinguishable by universally positive and negative traits, further studies with more strains and additional traits are necessary before these clusters can be taken species designations.

Mosquiotocidal strains of *Bs* can be divided into two groups on the basis of their toxicity to mosquito larvae (Table1.1). Strains which are highly toxic make parasporal crystals, whereas the strains with low toxicity lack a crystal. The high- and the low toxicity strains are related by DNA homology values of over 79%, a finding consistent with their placement into single species (Krych *et al.*, 1980). These strains have been placed into DNA homology group IIA, which is 57 to 66 % related to DNA homology group IIB, consisting of nontoxic strains. Although only a few diagnostic traits are useful for their identification, these two closely related groups have also be differentiated by a numerical analysis of their phenotypic properties (Alexander and Priest, 1990).

Table 1.1 Properties of the most extensively studied mosquitocidal strains of *Bs*

Crystal	Toxicity	DNA Homology Group	Flagellar serotype	Phage group
-	L	IIA	1	1
-	L	IIA	2a2b	2
+	H	IIA	5a5b	3
+	H	IIA	5a 5b	3
+	H		5a5b	3
+	H		25	4
+	H		5a5b	
+	H		6	3
+	H	IIB	6	

The larvicidal strains of *Bs* share number of properties. All are Gram positive, strict aerobes an unable to use glucose, denitrifying, or reduce nitrate to nitrite. No one is able to utilize pentoses, hexoses, or disaccharides as the source of carbon and energy and all can utilize gluconate. All strains grow at 30^{o}C and have growth factor requirements which may be satisfied by biotin and thiamine (Yousten, 1984).

In the course of sporulation, some strains of *Bs* synthesize a parasporal inclusion or "crystal", which contains proteins toxic for larvae of variety of mosquito species. Upon completion of sporulation, the crystal remains associated with endospore, both being enclosed within exosporium. The major components of the crystal are two proteins of 51- and 42-kDA, called BinA and BinB respectively (Baumann *et al.*, 1985). Neither protein alone is toxic to larvae, and

both are required for toxicity. For purified crystals from Bs, the dose that can be kill 50% of a mosquito larval population within 48h (LC_{50}) is 1-5 ng protein.ml^{-1}.

The crystal of *Bs* is a parallel piped. The interior shows a crystalline lattice structurewith striations about 6.3 nm apart (Yousten and Davidson, 1982). The crystal is surrounded by an envelope. The envelope appears to be retained upon dissolution of crystal matrix in the larval gut or after treatment with alkali.

The relation between growth, sporulation, crystal formation and toxicity for mosquito larvae has been studied for strains 1593, 2297, and 2362 (Broadwell and Baumann, 1986). The 51- and 42-kDa proteins were absent in the exponential phase of growth and appeared in approximately equal amounts during sporulation (de Barjac *et al.*,1988). The association between crystal formation and sporulation was further studied with oligosporogenic mutants. Mutants blocked at early stages of sporulation failed to make crystal proteins and were nontoxic, whereas mutants blocked at late stages of sporulation contained crystal proteins and were toxic (de Barjac *et al.,* 1988).

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of potentially purified crystal preparations from strain 2362 indicated that major constituents were proteins of 122, 110, 51, 42 kDa (Baumann *et al.*, 1985). The 122- and 110-kDa proteins were subsequently shown to be contaminants originating from the surface layer proteins of *Bs* (Bowditch *et al.*,1989).

The N- terminal sequence of 40 amino acids of the 42-kDa protein was dtermined; the 51 kDa protein had a variety of N termini thereby precluding sequence determination. Using synthetic oligonucleotide probes designed on the basis of the N-terminal sequence of the 42 kDa protein (Baumann *et al.* 1985) and genomic library of *Bs* 1593 (Hindley and Berry, 1987) cloned the gene for the 42-kDa protein into *E.coli* and determined its sequence.Baumann *et al,.* (1988) cloned into *E.coli* and sequenced a 3.5-kb *Hind* III fragment from strain 2362 which contained the genes for 51- and 42-kDa proteins. Subsequently, the sequence of a similar DNA fragment was determined from four additionally highly toxigenic strains (Berry *et al.,* 1989).

There is no defined evidence for location of crystal genes either on plasmids or on chromosome. One highly toxic strain lacked detectable plasmids, an observation suggesting that the toxin genes are chromosomal. Singer (1987) found a 75-Mda plasmid in many larvacidal and nonlarvacidal strains.

The most extensive study of the changes in the larval midgut epithelium following ingestion of sporulated *Bs* cells was that of electron microscopy study of Charles (1987). A number of studies have established that action of the crystal toxin on susceptible larvae involves the following series of steps : 1) Ingestion of crystal-spore complex 2) Solubilization of toxin in the midgut by alkaline pH 3) Processing of the 51- and 42-kDa, proteins to 43- and 39-kDa proteins, respectively. 4) Binding of toxin proteins to the cells of the gastric cecum and

posterior midgut and 5) Exertion of the toxic effect by means of an unknown mechanism.

Since the crystal proteins are processed to a lower molecular weight form in the midgut, it is not possible to use larval bioassays to determine whether the higher molecular weight precursors are toxic. One solution to this problem is to use of tissue culture grown cells. The cell line most susceptible to the *Bs* toxin and most commonly used has been derived from ovarian tissues of *Culex quinquefastaciatus* (Hsu *et al.,* 1970). The summary of the processing of the crystal proteins in mosquito larvae and toxicity of proteins to larvae and mosuito tissue culture grown cells is shown presented in Figure 1.1.

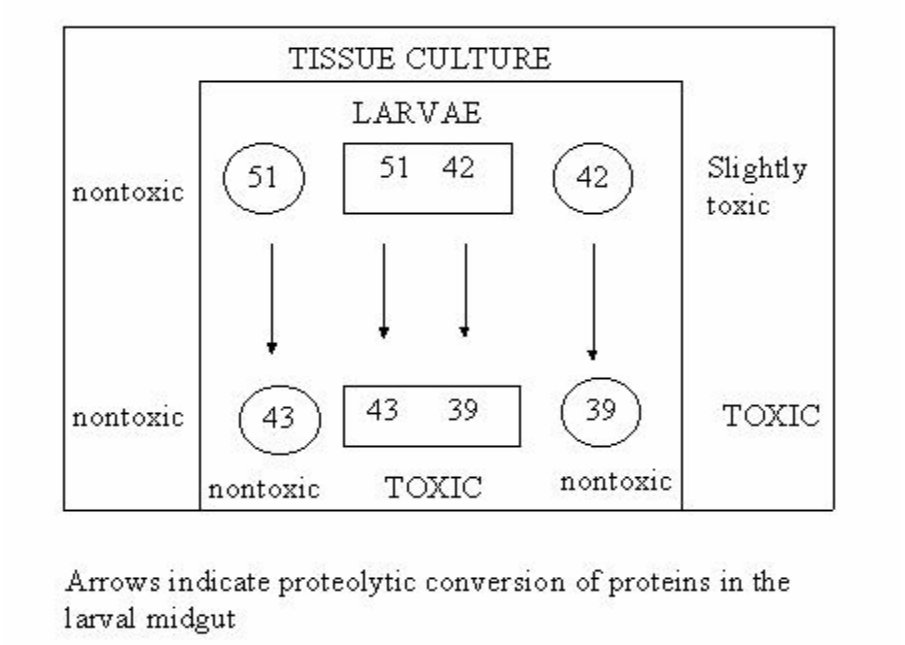


Figure 1.1 Processing of protoxins in larva and tissue culture.

Following ingestion of a mixture of spores and toxin-containing paraspores, the number of viable spores in the gut decreases until the death of the larvae which usually occurs within 24h after ingestion of the toxin. At that time, some unknown fraction of the spores germinate, grow vegetatively, and sporulate in the larval cadaver. This results in about a 10 to 100-fold increase in the number of spores compared to the number originally consumed (Charles and Nicolas, 1986; Davidson *et al.* 1984). This recycling of the spores in the cadaver may be related to the prolonged larval control reported for *Bs* but not for *Bti* (Singer 1987). Recently, persistence of *Bs* in environments subject to exposure to UV light, acidic pH, cemical rsesidues and increased tempertaures were significantly

improved by entrapment in calcium alginate microcapsules (Elçin, 1995; Çökmüş *et al.,* 2000).

In general, *Bs* is more active against *Culex* and *Anopheles* species and less active against *Aedes* species, whereas Bti is more active against *Aedes* and *Culex* species and less active against *Anopheles* species (Lacely and Undee 1986). *Bti* is also active against blackfiles whereas *Bs* is not. *Bs* has an additional and useful attribute that it is able to persist in polluted aquatic environments in which the toxicity of *Bti* is rapidly lost.

The mechanism of action of insecticidal *Bacillus* toxins involves a number of steps, which was considered to be an advantage over synthetic insecticides. However, resistance under laboratory and field conditions has been reported for *Bs.* Different levels of resistance to Bs have been reported in *C.pipiens* populations (Rao *et al.,* 1995; Silivia-Filha *et al.,* 1995; Yuan *et al.,* 2001). The level and stability of resistance and time needed for its development, depend on various factors, including genetic background, selection pressure, the insect generation turnover, migration level and, most importantly, the genetic dominance of resistance. In all studided cases, resistance seems to be due to recessive trait (Wirth *et al.,* 2000), which is consistent with the reversion of resistance under field conditions (Rao *et al.,* 1995; Silva-Filha *et. al.,* 1997 Yuan *et. al.,* 2000). Resistance is due to one major sex-linked gene in the field resistant *C.pipiens* colony in France (Nielson-Le Roux; *et al.,* 1997), and to one major and

probably autosomic gene in a laborator-selected resistant colony from California (Wirth *et al.,* 2000).

Until now, only one mechanism of resistance has been revealed. However, *in vitro* toxin-receptor bindidng experiments conducted with larval midgut isolated from *Bs*-resistant colonies showed that, in some cases, the receptor was not functional, but in others, this interaction was similar to susceptible colony (Nielson-Le Roux; *et al.,* 1997). The facts taht there are at least two possible mechanisms of resistance, arising from two different mutations, and that either one is sufficient to decrease the larvacidal efficacy might explain why resistance to *Bs* likely to occur.

In terms of safety and environmental impact, WHO (World Health Organization) has established a five satge evaluation system to which potential biological field candidates must submit (WHO- Geneva 1989). In addition fate of *Bs* 2362 spores following ingestion by nontarget vertebrates have been studied by Yousten, *et al* (1991).This research team have concluded that nontarget organisms are unaffected but exposed nontargets might tarnsport microbial pest control agents to untreated sites. Although to date there appears to be no adverse effects in terms of mammalian toxicity, *Bs* might cause central venous catheter (CVC) bacteraemia in immunocomprimised children (Castagnola *et al.,* 2001).

Recent studies about larvacidal *Bs* strains concentrated on much more difficult tasks: 1) Improvement of *Bs* toxixcity by genetic engineering 2) Understanding the function of the components of the binary toxin, the mechanism of the toxin action.

In order to improve toxicty of *Bs* or co-expression of larvacidal *Bt* genes, genetic engineering was prefered by a number of researches. Poncet *et al*. (1997) tried to improve *Bs* toxicity against dipteran larvae by integration via homologous recombination, of the Cry11A toxin gene from *Bti*. The results of that study appears to be promising to creation of new *B. sphaericus* strains for vector control. Similarly, co-expression of *cyt1Aa* of with *Bs* binary toxin gene in acrystalliferous strain was studied (Zhang *et al.,* 2000). In the strain constructed in this way Cyt1Aa protein and binary toxin proteins possibly interacted synergistically, thereby increasing its mosquitocidal toxicity significantly.

The studies around second difficult task which involves understanding of the function of the components of the binary toxin and action mechanisms has been provided new approaches and solutions. In this context, site directed mutagenesis experiments were performed for identification of the functional site of larvacidal binary toxin in mosqito (Jayarman *et al.,* 2000). Membrane biology of target cells also studied extensively, especially in recent years. Davidson *et al.,* (1997) concentrated on electrophsiological effects of *Bs* binary toxin on cultured mosquito cells. Another approach was permeabilization of model lipid

membranes by *Bs* mosquitocidal binary toxin and its components (Schwartz *et al.*, 2002) .

1.2.*Bacillus thuringiensis* and Delta-Endotoxins (Crystal Proteins)

The discovery of leading rational pesticide, *Bacillus thuringiensis (Bt)*, is credited to Ishiwata. In Japan, in 1901, he isolated the organism responsible for flacherie, a disease of silkworm larvae (Bmbyx mori) and named it *Bacillus sotto.* In 1911, a similar *Bacillus* was isolated by E. Berliner from diseased larvae of Mediterranean flour moth (*Anagasta kühniella)*. Berliner named his isolate *Bt* after the province of Thüringen, in which it had been discovered. This isolate was Gram (+), spore-forming, rod shaped soil bacterium. Numerous strains of *Bt* have been described since that time and each has its own distinct spectrum of pathogenic effects on its host insects.

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* which was used for the control of the insect (Lepidopteran) pests in agriculture and forestry (Lüthy *et al.*, 1982). 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 protein or delta endotoxins. The genes encoding for the crystal protein 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 after the sporulation is completed. Also a cytolytic protein, cytolysin (Cyt), is found in the crystal inclusions of the Diptera active strains.

Bt strains can be isolated world wide from many habitats, including soil, insects, stored product dust, and deciduous and coniferous leaves (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 endotoxin composition is the parameter that determines the range of insect pests on which the organism is active.

In 1989, Höfte and Whiteley reviewed 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, beneath 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 40th 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 1.2 shows the types of crystal proteins and the insect orders to which they are active.

Table 1.2. 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

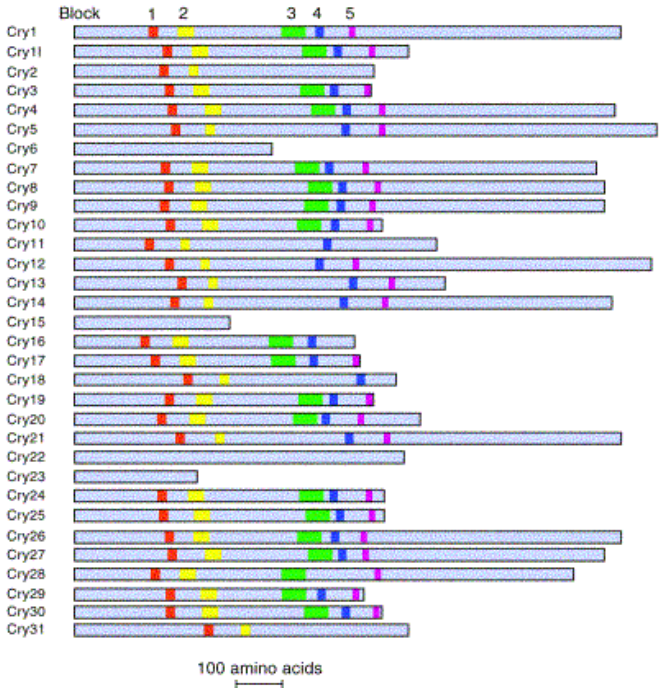


Figure 1.2. Conserved sequences of insecticidal crystal proteins.

Shown in Figure 1.2. 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 1.2 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 remarkable diversity of *Bt* strains and toxins is due to at least in part to high degree of genetic plasticity. *Bt* strains have genome size of 2.4 to 5.7 million bp (Carlson *et al.*, 1994). Most *Bt* toxin genes appear to reside on plasmids (Gonzalez *et al.*, 1981), often as parts of composite structures that include mobile genetic elements (Kronstad and Whitely, 1984). Many *cry* gene containing plasmids appear to be conjugative in nature (Gonzalez *et al.*, 1982). A common characteristics of *cry* genes is their expression during sporulation phase. Their products generally accumulate in mother cell compartment to form crystal inclusion that can account for 20 to 30% of dry weight of sporulated cells.The very high level of protein synthesis in *Bt* and its coordination with stationary phase are controlled by number of mechanisms at transcriptional, posttranscriptional and post tranlational levels (Agaisse and Lereclus, 1995; Baum and Malvar, 1995).

To date, the structures of three crystal proteins- Cry3A, Cyt2A (Li *et. al.,* 1991) and Cry1Aa (Grochulski et al., 1995)- have been solved by X-ray crystallography.

The crystal proteins are synthesized as protoxins that are not active. Protoxins possess three domains. The long hdrophobic and amphipatic helices of domain I suggest that this domain might be responsible for the formation of lytic pores in the intestinal epithelium of target. This domain bears many similarities to the pore forming or membrane translocating domains of several other bacterial

toxins including colicin A, diphteria toxin (Parker and Pattus, 1993). The surface exposed loops at the apices of three β-sheets of domain II, because they show similarities to immunoglobulin antigen binding sites and it is known to recognize the carbohydrate moieties of its receptors (Knowles *et al.,* 1984; Schnepf *et al.,* 1998). The β sandwich structure of domain III could play a number of key roles in the biochemistry of toxin molecule. Li *et al.,* (1996) sugget that domain III functions in maintaining the structural intgrirty of the toxin molecule by protecting it from proteolysis within the gut of the target organisms. Figure 1.3 illustrates the 3D structure and the domains of the crystal proteins.

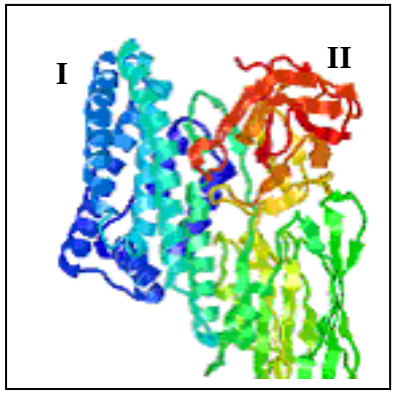


Figure 1.3. 3D structure and the domains of crystal protein Cry1A.

The mechanism of action of the Cry proteins involves synergistic interaction of four toxins. Binding of toxin molecules to specific membrane receptors, or to membrane phospholipids (for Cyt proteins) (Haider and Ellar, 1989) is the first step of the formation of cytopathological effects of protoxins. A part of toxin inserts into the membrane and forms a transmembrane channels. For the protoxin to become active they are solubilized under the alkaline conditions of the insect midgut to become activated (Hoffmann *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). The major proteases of the insect midgut are chymotrypsin-like or trypsin-like proteases (Lecadet and Dedonder, 1966; Johnston *et al.,* 1995; Peterson *et al.,* 1995; Novillo *et al.,* 1997). The initial molecular weight of the Cry1A protoxin 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 susceptible insect (Schnepf *et al.,* 1998). In seveal insects- *Manduca sexta*, *Lymantria dispar* etc.- there are aminopeptidase enzymes which may possess more than one binding site (Masson *et al.,* 1995; Jenkins *et al*., 2000). In addition, they have cadherin-like proteins functioning as receptors for Cry toxins (Nagamatsu *et al.*, 1998; Gahan *et al.,* 2001; Griffitts *et al.*, 2001; Dorsch *et al.,* 2002). Binding is a two stage process, involving

reversible binding and irreversible binding. The reversible binding is performed by binding of domain II to the receptor. This binding involves a tight binding between the toxin and receptor. It 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). On the other hand irreversible binding is exclusively associated with membrane insertion. This irreversible binding has not yet been well understood (Duche *et al.,* 1994; Lesieru *et al.,* 1997; Schwartz *et al.,* 1997).

After insertion into the membrane and pore formation, an influx of the water with the ions occurs, that causes the 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). Figure1.4. summarizes the mechanism of toxicity of *Bacillus thuringiensis*.

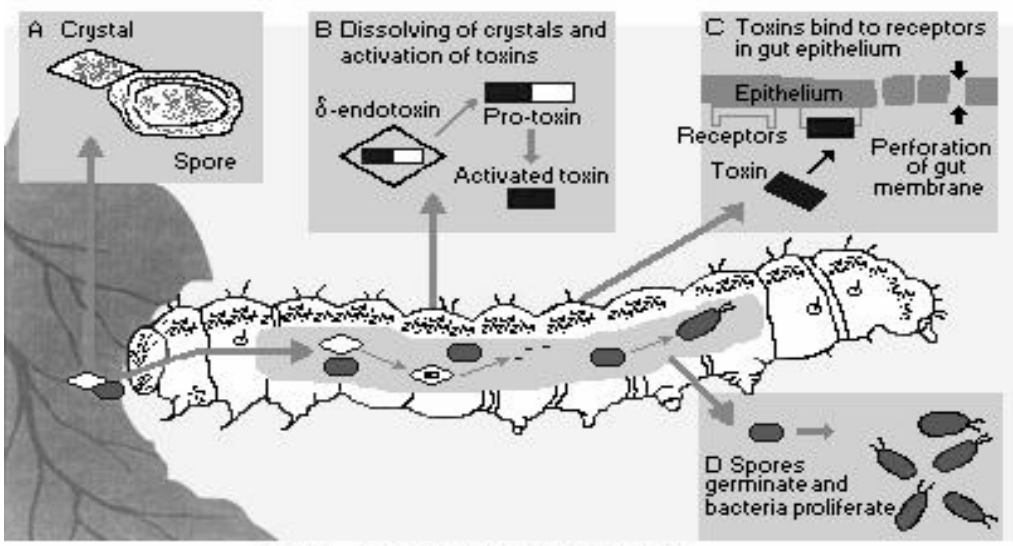


Figure 1.4. Mechanism of toxicity of *Bt.*

Beta-exotoxin is associated with certain *Bt* subspecies (*Btd, Btg, Btte and Btt*), and products made from these *Bt* subspecies may contain the toxin. This heat-stable nucleotide, which is composed of adenine, glucose and allaric acid, inhibits RNA polymerase enzymes by acting competitively with ATP (Farkas et al., 1977). Since RNA synthesis is a vital process in all life, beta-exotoxin exerts its toxicity for almost all forms of life tested including numerous insect species in the orders *Coleoptera, Diptera* and *Lepidoptera.* Bt containing beta-exotoxin is used for the control of houseflies in some countries, but regulatory agencies currently prohibit the use of beta-exotoxin for other purposes.

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 *Bacillus thuringiensis 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.

A number of insect populations of several different species with different levels of resistance to *Bt* have been obtained by laboratory selection experiments during the last 15 years (Schnepf *et al.,* 1998). The species include *Plodia interpunctella, Cadra cautella, Leptinotarsa decemlineata, Chrysomela scripta, Tricholplusia ni, Spodoptera littoralis, Spodoptera exigua, Heliothis virescens, Ostrinia nubilalis* and *Culex quinquefasciatus* (Schnepf *et al.,* 1998) and resistance is shown to *either Btk, Bti, Btte* or other *Bt* subspecies.

During the last few years populations of the diamondback moth, *Plutella xylostella,* resistant to *Btk* and *Bta* have been found in heavily treated areas in

numerous geographically isolated regions in the world, including Hawaii, Phillippines, Indonesia, Malaysia, Central America and some USA states (Schnepf *et al.,* 1998). It is clear that this widespread appearance of resistance to *Bt* presents a cautionary tale for the way of using *Bt* and *Bt* toxin genes in pest management. Schnepf *et al.,* (1998) have reviewed resistance management of *Bt.*

Bt has evolved to produce large quantities of crystal proteins, making it a logical host for developing improved Cry biopesticides. Natural isolates of *B. thuringiensis* produce several different crystal proteins, each has different target specificity. However, certain combinations of Cry proteins have been shown to exhibit synergistic activities (Wu *et al.,* 1994; Crickmore *et. al.,* 1995). Thus, genetic manipulation of *Bt* may be desirable. A number of transconjugant have been registered with the U.S. Environmental Protection Agency. Then in 1989 engineered *Bt* and *B.cereus* came. Several groups independently applied electroporation technology to transform vegetative cells with plasmid DNA (Belliveau and Trevors, 1989; Bone and Ellar, 1989). A variety of shuttle vectors, some employing *Bt* plasmid replicons has been used to introduce cloned *cry* genes (Chak *et al.,* 1994; Baum *et al.,* 1990). Alternatively integration vectors have been used to insert cry genes by homologous recombination into resident plasmid or the chromosome (Lereclus *et al.,* 1992; Adams *et al.,* 1994). Recently asporogenous crystal producing *Bt* strains have been developed (Bravo *et al,* 1992; Lereclus *et al.,* 1995). These crystals remain encapsulated in mother

cell compartment. Although it is untested, this property might improve toxin persistence in field applications.

The strain improvement strategies have been accelerated recently. The purpose of strain improvement is 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. 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.

As more knowledge gained in *Bt* genetics, alternative delivery systems for Cry proteins have been developed. The *cry2Aa* gene from *Bt kurstaki* has been expressed in an endophytic isolate of *Bacillus cereus* (Mahaffee *et al.*, 1994). And *Bacillus megaterium* isolate that persists in the phyllosphere has been used as a host for *cry1A* gene (Bora *et al.*, 1994). Similarly, cry genes have been transferred into other plant colonizers, including *Azospirillium* spp., *Rhizobium leguminosarum*, *Pseudomanas cepacia* (Skot *et al,* 1990). Baculoviruses (e.g.

Autographa californica), the naturally pest occuring pathogens have also been employed as an expression system for *cry* genes (Je *et al*., 1997). Alternative delivery systems have also been sought for dipteran active toxins of *Bt israelensis* to increase their persistence in the aquatic feeding zone. Such host include *Bacillus sphaericus* (Bar *et al.,* 1991), *Caulobacter crescentus* (Thanabalu *et al.,* 1992) and cyanobacteria (Soltes *et al.,* 1993).

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).

1.4. About Target Organisms : Lepidoptera and Diptera

Lepidoptera

Lepidoptera constitute one of the largest order of insects with over 100,000 species worldwide. Lepidoptera are Holometabolous insects (insects which have larvae that look nothing like the adults and having a complete metamorphosis with pupal stage. All undergo a complete form of development (egg, larva, pupa, adult). Lepidopteran larvae have chewing mouth parts with strong

mandibles. Most adults have long, coiled, tube-like or straw-like mouthparts which are used for sucking up plant nectar. They possess two pairs of membraneous wings with few cross veins (though these may be absent in the females of some moths). The antennae are variable in length and may be quite complicated in some male moths. They have two large compound eyes with as many as 6000 omatidium and two ocelli, while the larva often have simple ocelli. Lepidoptera normally have rather hairy bodies, long antennae and their wings are covered with tiny scales. These scales reflect the bright colour patterns of the wings and form eyespots in many moths. The salivary glands of the larvae have become modified to form the silk glands. The larvae are 'eruciform' (which means they look like a caterpillar) and in most cases have 13 body segments. The larvae, which are called caterpillars, eat plants and often cause considerable damage to forests, gardens, and croplands are the forest tent caterpillar, *Malacosome disstria* and the gypsy moth *Lymantria dispar.*

Diptera

The Diptera are commonly known as (true) flies and include many familiar insects such as mosquitoes, black flies, midges, fruit flies, blow flies and house flies. Flies are generally common and can be found all over the world except Antarctica. Many species are particularly important as vectors of disease in man, other animals, and plants. In addition, much of our knowledge of animal genetics and development has been acquired using the vinegar fly Drosophila melanogaster (family Drosophilidae) as an experimental subject .

The earliest fossil flies are known from the Upper Triassic of the Mesozoic geological period, some 225 million years ago. Since that time they have diversified to become one of the largest groups of organisms. There have been about 120,000 species of flies formally described by scientists; thus about 1 in every 10 animals described is a fly. An equal number of species may await description and most of these will be found in environments that remain to be studied intensively, such as tropical forests.

Flies are holometabolous insects, that is their life cycle involes a major change in form from a soft-bodied, wingless larval stage to a hardened, winged adult. Larval flies have a variety of common names, such as wriggler and maggot. Fly larvae have an enormous variety of feeding habits, and individual species often have very precise requirements. Many consume decaying organic matter, or are predacious, and a large proportion are parasitic on other insects and other organisms. Adult flies are almost always free-living and do fly during the day. They typically consume liquid food such as nectar and other plant exudates, or often decomposing organic matter. The major morphological feature which distinguishes flies from other insects is their reduced hind wings, termed halteres. The halteres are small, club-like structures that function as balancing organs during flight. Thus adult flies have only one pair of functional wings from which their scientific name is derived Diptera (di - two, pteron - wing). A few other groups of insects have also convergently attained a similar two-winged

form, such as male coccoids (Hemiptera-Sternorrhyncha). A few flies have lost their wings (and halteres) altogether.

Because of the reliance on the forewings for flight, the mesothorax has become enlarged to contain the enormous flight muscles, and the pro- and metathorax are correspondingly reduced. The mouthparts of flies are also characteristically suctorial and many have large fleshy pads with drainage canals termed pseudotracheae for efficient liquid uptake. Some flies have mouthparts modified for stabbing and piercing other insects, such as the predatory robber-flies (Asilidae) and dance flies (Empididae). Mosquitoes and some other ectoparasitic groups have mouthparts modified for piercing the skin of a vertebrate host and removing blood and other fluids.

Larval Diptera are typically small, pale and soft-bodied. They lack true legs and move by peristaltic waves of muscular contraction through the body. The larvae of most species of flies have a reduced head capsule and all that remains are the mandibles and some associated sclerites which are collectively called the cephalopharyngeal skeleton.

1.5. The Concept of Local Production

The term local production of microbial insecticides refers to the development of production (fermentation and formulation) facilities in developing countries, in contrast to the existing production facilities in more industrialized nations.

There are number of advantages in promoting development of local production facilities for larvacides.The most important advantage of local production concerns stability. One of the disadvatages of using microbial agents to control pests has been instabilit and the variaton of the toxicity of the formulations. This lack of stability was most likely the result of the lengthy shipping periods and long and variable storage temperatures before the product reached the consumer. To avoid these instability and varibility problems with microbial larvacidal agents, local production should be encouraged.The second advantage of local production concerns appropriate formulations. The deelopment of such formulations will depend largeley on the result of actual field-trial data. Due to very different environmental conditions between tropical or dry countries and temperate industrial countries. Thus, there will be no single formulation that will be effective for all field conditions and local production of microbial agents would be beneficial in providing material for conducting appropriate field studies and for developing formulations suitable for local environmental conditions. The last advantage of local production concept is the dependency on

the locally available, cheap raw materials.Table1.3. lists the preferable raw materials used in local production trials.

Table 1.3. List of Potential Raw Materials for Use in Formulating Fermentation Media for Local Production of *B.sphaericus* and *Bti.*

Raw Material	**Comment - Reference**
Agricultural Products	
Bambara bean, cowpea	Obeta and Okafor 1983
grounnut cake, soybean, gruel	Zouari and Jaoua 1999
Ground corn,cotton seed meal,	Vandekar and Dulmage 1983
cassava, yams	
Animal Products	
Blood, beefbones, chikenparts, fish meal	Hertlein et al. 1981,Vandekar and Dulmage 1983
animal dung, broiller litter extract	Vandekar and Dulmage 1983
	Adams *et al.,* 1999
Industrial Byproducts	
By product from monosodium glutamate	Dharmshiti *et al.,* 1985.
factory, wastewater sludge	Vidyarthi *et al,.* 2002
Other	
Coconut milk, cornsteep liquour, molasses	
Whey	Sasaki *et al.,* 1998

In summary, local production of biological larvacides would not only allow the development of general fermentation capability in developing countries, but would also provide more stable and more suitable product formulation i.e. with cheaper raw materials, shorter shipment period, shorter shelf-liferequirement, and better qlity of low-cost flowable formulations. Nonetheless, it appears that to

achieve the aim of local production capability, the final price of the product has to be competitive with the commercially available products.

In this study, we attempted to optimize lab-scale production of entomopathogenic crystal toxins of three different biolarvacidal strains prior to stage of local industrial production.

Bacillus sphaericus strain 2362 (*Bs*) was chosen as the first candidate due to its good persistence and recycling capacity in organically polluted water habitats. In the industry, research for better carbon and nitrogen sources is essential activity, since the carbon nitrogen source is usually the highest cost ingredient of the medium. Thus, growth of *Bs* 2362 was tested as the functions of carbon-nitrogen source and incubation time.

The effects of various nutritional and cultural factors for a local isolate *Bacillus thuringiensis* subsp. *kurstaki* 81 (*Btk* 81) crystal toxin components Cry1Aa (135 kDa and Cry2Aa (65 kDa) have been repoted by our laboratory (İçgen et. al., 2002). On the basis of these findings, the effects of carbon to nitrogen ratio (C:N ratio) on the crystal protein production efficiency is aimed to be investigated in this strain.

In the last part of the study the efficiency of *Bti* ONR60 was tried to be evaluated as the function of C:N ratio. Optimized conditions tested in pilot scale

production trial for *Bacillus thuringiensis* ONR60 based bioinsecticide in 30L batch type fermentor

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial Strains and Their Maintenance

The three larvacidal strains used throught the study were: *Bacillus thuringiensis* subsp. *kurstaki* 81-1, *Bacillus sphaericus* 2362, *Bacillus thuringiensis* subsp.*israelensis* 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 of Stahly *et al.*, (1992):

Bacterial strains were grown 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 than 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 coressponding medium and grown by shaking. When the culture reached to mid-log phase, a required amount from this culture (seed culture) was transferred to fresh medium.

2.2. Culture Media

Thus B*tk* 81-1 was grown in YSM (Yousten and Roegff, 1969), *Bs* 2362 was grown in NYSM (Myers and Yousten, 1980) and *Bti* was grown in TBL (US Patent Reference, Patent number:4448885). The composition and preparation of these media are given in Appendix A.

2.3. Buffers

The buffers used 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 medium 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 at 2 h intervals and used for quantitative determination of growth which was measured spectrophotometrically at 600 nm. Viable counts

were also made and cell concentrations were determined as “colony forming units/mL” for both the strains simultaneously.

2.6.Crystal Protein Staining

Crystal proteins were identified as in Sharif and Alaeddinoğlu, N.G. (1988). Comassie brillant blue solution was poured to cover dried and fixed bacterial smears for 3 min. The preparation was washed with dH_2O and dried. Light microscope (100X magnification) used to observe crystal proteins and spores.

2.7. Enumaration of Heat Resistant Spores and Determination of Sporulation Frequency

Heat resistant spores were enumarated as in Özcengiz and Alaeddinoğlu(1991). 1 mL portions of cultures were serially diluted in physiological saline tubes. The tubes were heated at 85°C for 20 min in a water bath. The tubes were cooled and dilutions were plated into nutrient agar. Colony forming units were determined after an overnight incubation at 30°C. The incidence of heat resistant spores (sporulation frequency) was expressed in terms of the ratio of the number of heat-resistant spores per mL to the ratio of the number of viable cells per mL.

2.8. 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 waterbath 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 (Bradford, 1976).

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.

2.9. 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).

2.10. Staining of SDS-Polyacrylamide Gel

Staining of SDS-polyacrylamide gels was performed as follows (Baum *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	

2.11. Protein Quantification

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

2.12. Determination of Glucose concentration by Anthrone Reaction

A slightly modified procedure of Morris, D. L. (1948) was used for protein extraction. Prior to glucose determination analysis a standard curve was developed by using stock glucose solution [100 mg glucose was dissolved in 100 mL of 0.15 % (w/v) benzoic acid. It can be stored at 5 °C for several months]. This solution was diluted 10 times in dH_2O before use to give a solution containing 100µg /mL. Standart solutions were prepared at the range of 10 to 100 µg /mL. 10 mL of samples were taken from the bacterial culture at 12 h intervals and filtered through a 0,2µm filter disc. Filtrat was next pipetted into the tubes and diluted with dH_2O appropriately. 1 mL of this was transfered into a boiling waterbath. After 5 minutes, tubes was chilled in an ice-water bath. 5 mL of freshly prepared chilled anthrone reagent was (200 mg anthrone was dissolved in 5 mL of absolute ethyl alcohol and the volume completed to 100 mL with 75 % H_2SO_4 solution. Stored at 5 °C) was added into the tube. Tube content were mixed rapidly by swirling the tubes in ice water bath for 5 min. Then, tube was transfered to a bath of boiling water and kept these for 10 min.

After cooling on ice, absorbance was read at 625 nm and glucose concentration was determined by using of standart curve. dH_2O was used as the blank reagent.

2.13. Determination of Nitrogen Concentration

10 mL of samples were taken from the bacterial culture with 12 h intervals and filtered. Nitrogen measurement in culture fluid was performed by Merck-Spectroquant Nitrogen Cell Test kit.

2.14. Pilot Scale Production Trial

Pilot scale production of *Bti*-based bioinsecticide was performed for *Bacillus thuringiensis* subsp. *israelensis* ONR60 strain. For this aim, all of the facilities of the factory was kindly provided by Dr. Mehmet BATUM (ORBA Biochemicals Inc.) Prior to the fermentation fermentor was sterilized two times at 121^{o}C for 1 hour. The working temprature was set to 30^{o}C. Oxygenation rate was 0.75 v/v/m (0,75 volumes of air per volume culture suspension per minute). Sampling was achieved at 8 hours intervals. Samples collected were used to check optical density, growh and sporulation behaviors, pH alteration, carbon and nitrogen utilization characteristics and protein profile of bacterial culture.

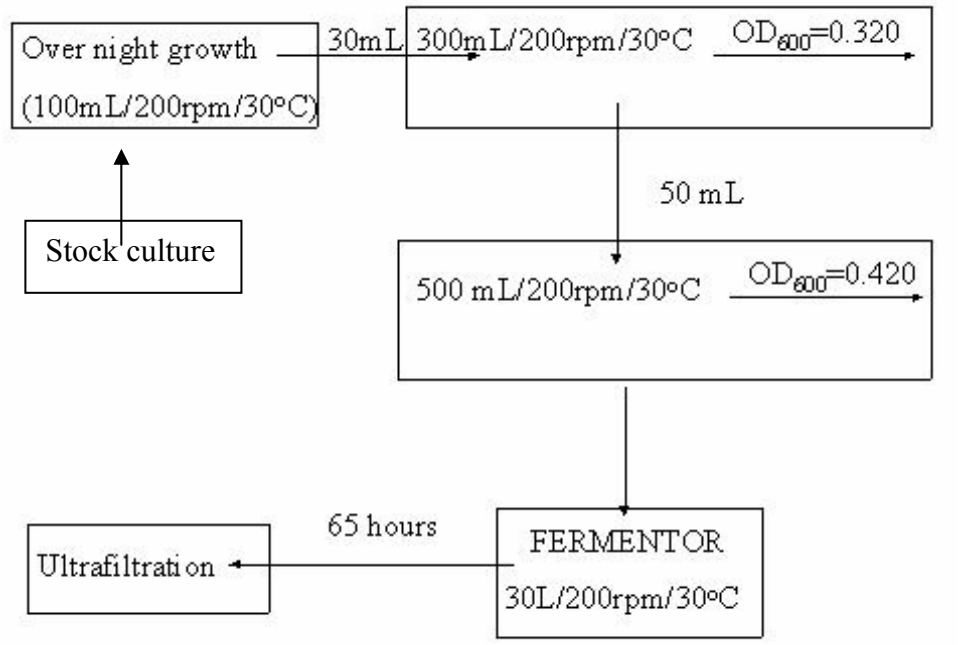


Figure 2.1. Schematic description of inoculum preperation,fermentation and down stream.

2.15. Bioassay analysis

Bioassay analysis was performed by Dr. Öner Koçak, Hacettepe University, Faculty of Education, Division of Biology Education. Second to third instar *Culex* sp. larvae were used in bioassay. 15 to 35 larvae were placed in each plastic container containing sterile tap water. Samples obtained after centrifugation of the culture were added at the series of dilutions, to the bioassay cups at 30^{o}C. Mortality was assesed at 24 and 48 h following inoculation against a standart (Abott).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Growth of *Bacillus sphaericus* strain 2362 as the Functions of Carbon Source and Incubation Time

As mentioned earlier, *B. sphaericus* 2362 (*Bs* 2362) produces binary toxins designated BinA (51 kDa) and BinB (42 kDa). These toxin proteins 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 those for growth of the producer cells. In many of the previous studies NYSM (Nutrient Broth Yeast Extract Sporulation Medium) have been preferred for the production of *Bs* (Myers and Yousten, 1980; Campbell *et. al.,* 1991; Elçin and Çökmüş 1995). Yeast extract and nutrient broth are the main carbon and nitrogen source of that medium. It acts as the source of amino acids and vitamins for both growth and sporulation. However, yeast extractand nutrient broth are relatively expensive. Thus, most readily available and least expensive substrates were considered prior to cost effective large scale local production trials. In order to make an efficient choice, carbohydrate metabolism characteristics of *Bs* strains

are considered. Although no *Bs* starins able to utilize pentoses, hexoses, or disaccharides as source of carbon and energy, they have the capacity to metabolize proteinaceous materials.

Accordingly, in our the study on the production of *Bs* 2362, we mainly focused on whey and soy powder based media. Whey is the watery portion or serum of milk that is separeted from curd during conventional cheese or casein manufacture. It retains about 85 to 90 % of the volume of milk used, and about 55% of the milk nutrients. These include lactose, whey proteins, mineral salts and vitamins.Also, soybean flour has a high protein content. The protein content of the soybean flour used in this study was 70%. When both whey and soybean flour based media were reformulized, yeast extract and nutrient broth content of NYSM was replaced with whey and soybean flour, respectively. Other components of the medium were not changed. These reformulized media were renamed as WYSM and SYSM, respectively, to refer the carbon and nitrogen sources they contain (Appendix A). Their efficiencies were compared in terms of cell density and spore production (Figure 3.1 and Table 3.1). In order to construct growth curves, viable cell count was performed. Because high insoluble particulate contents of the media did not allow the use of spectrometers, viable count was necessary for growth measurement.

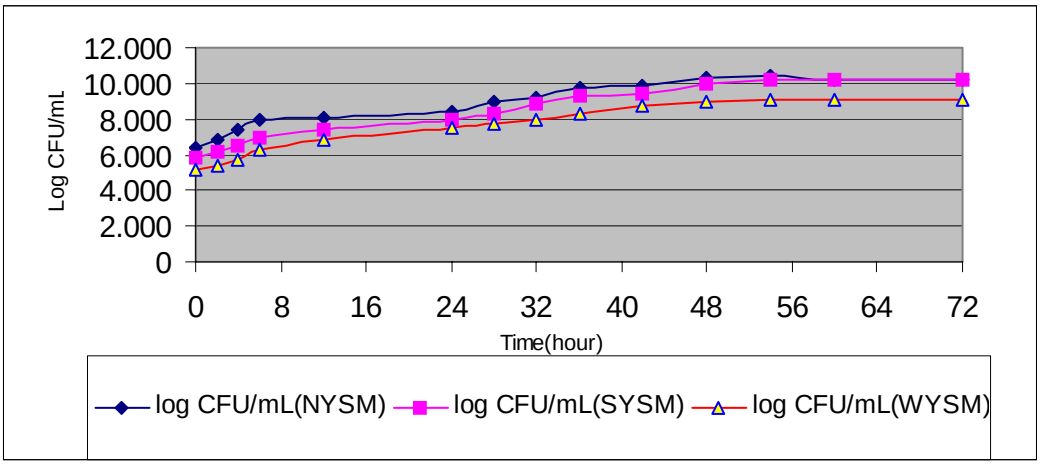


Figure 3.1. Growth curve of *Bs* 2362 in three different media.

Table 3.1 Effects of three different carbon and nitrogen substrates on sporulation frequency.

	Sporulation Frequency		
Incubation Time (h)	NYSM	SYSM	WYSM
12	$2.4x10^{-4}$	$2.1x10^{-4}$	$1.5x10^{-5}$
18	$3.2x10^{-3}$	$1.2x10^{-3}$	$3.4x10^{-4}$
24	$1.5x10^{-2}$	$2.8x10^{-3}$	$4.1x10^{-4}$
30	$2.2x10^{-2}$	$1.9x10^{-2}$	$3.2x10^{-3}$
36	$4.3x10^{-2}$	$2.8x10^{-2}$	$4.1x10^{-3}$
48	$1.2x\ 10^{-1}$	$3.2x10^{-2}$	$5.2x10^{-3}$
72	1.0	$1.1x10^{-1}$	$2.4x10^{-2}$

For comparison, the growth of *Bs* 2362 in NYSM, SYSM and WYSM was initially monitored against time (Figure 3.1). The growth of *Bs* 2362 was the best

in NYSM and fairly good in SYSM. However, its level in WYSM was below the levels in NYSM and SYSM.

Sporulation frequency was quantitavely determined at t_{12}, t_{18}, t_{24}, t_{30}, t_{36}, t_{48} and t_{72}, respectively (Table 3.1). While NYSM permitted the highest level of sporulation, sporulation was the lowest in WYSM and modarate in SYSM. Thus, the trends observed in growth were also observed in the sporulation frequencies.

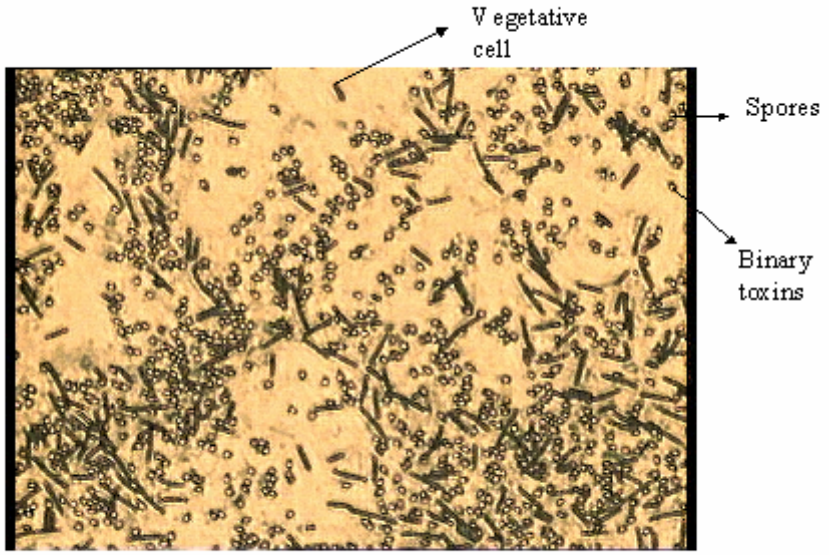


Figure 3.2. Micrograph showing vegetative cells, spores and binary toxins.

Spore and toxin formations were also checked microscopically. Figure 3.2 shows vegetative cells, spores and toxins in which had been taken from a 24 hour culture of *Bs* 2362 culture grown in SYSM.

Binary toxin synthesis was also investigated at different stages of growth to show its relationship with growth and sporulation. Protein extraction was done by taking samples at t_{24}, t_{48} and t_{72}. SDS-Polyacrylamide gel electrophoresis was performed for evaluation of binary toxin production by comparing thickness of the protein bands (Figure3.3). In view of the results, NYSM turned out to be the still the best choice, 72 hour being the most appropriate time of incubation. However the analysis of the protein bands also suggested that the SYSM might be an alternative to NYSM since the toxin protein bands in the samples taken at as early as 48^{th} hour were fairly thick in this medium at as early as 48^{th} hour band. A much shorter fermentation time for reasonable toxin yields is quite promising for large scale applications. Among the three media, WYSM seemed to be poorest one in respect to its capacity to support toxin production. These observations were also verified by the quantitative analysis of binary toxin concentration from the gel images (Table 3.2).

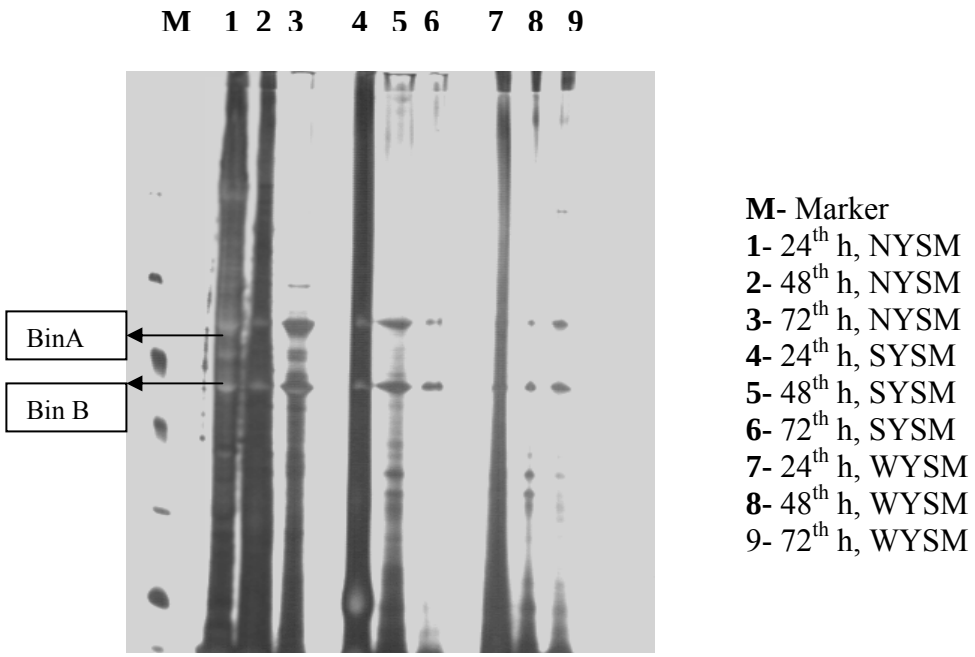


Figure 3.3. Production of binary toxins by *Bs* 2362 in three different.

Table 3.2. Concentrations of BinA and BinB binary toxins in three different media.

Binary toxin concenration (mg/mL)

Binary Toxin Type/Time (h)	NYSM	SYSM	WYSM
A/24	0.0038	0.0036	ND
B/24	0.0051	0.0047	ND
A/48	0.0048	0.0079	0.0021
B/48	0.0059	0.0092	0.0026
A/72	0.0102	0.0049	0.0034
B/72	0.0098	0.0052	0.0042

ND: Not Determined

Results obtained from these experiments showed that SYSM medium supported growth sporulation and toxin production at the levels comparable to those in NYSM medium. In addition, this newly developed medium was much less costly than that of NYSM medium for cultivation of *Bs* 2362. Yet, when the system is scaled up, some problems might be encountered. One problem can be related with the suspended solids in this medium. The solids content may form a mesh which decreases the adequate oxygen uptake by the cells. In addition, solids may interfere with the toxins during purification steps since the course particles will be carried over to the final product. Furthermore, if the toxin formulation is going to be a flowable or wettable powder, the particles might avoid the pipes and nozzles of spray apparatus. Thus, all these factors have to be re-considered prior to industrial applications. After that, the studies on the efficiency of binary toxin production might be revised or re-directed to more suitable choices.

3.2. Delta Endotoxin Production Efficiency of *Bacillus thuringiensis* subsp. *kurstaki* 81 Strain as the Function of Carbon to Nitrogen Ratio

A local isolate of *B.thuringiensis*, designated *B.thuringiensis* subsp. *kurstaki* 81 (*Btk* 81), was found to have strong antilepidopteran activity (Afkhami *et al.,* 1993). As stated earlier, industrial processes for crystal production are the most effective when high cell densities are achieved at high sporulation rates of more than 90%. Crystal protein synthesis was therefore investigated in association with growth and sporulation throughout the study.

The carbon:nitrogen ratio (C:N) is one of the important fermentation variable which has not yet been investigated. In order to investigate the effect of this variable [Cry1Aa (135 kDa and Cry2Aa (65 kDa)] various C:N ratios were applied to Yousten's synthetic medium since crystal proteins produced by *Btk* 81 have found to be the highest in this medium which also stimulated the sporulation (İçgen *et al.*, 2002).

Before studying the effect of C:N ratio, growth and sporulation of *Btk* 81 was determined at the conditions optimized by İçgen *et al.*, (2002) (Figure 3.4 and Table 3.3). It is to be noted that in this optimized YSM, the C:N ratio is about 1.5:1.

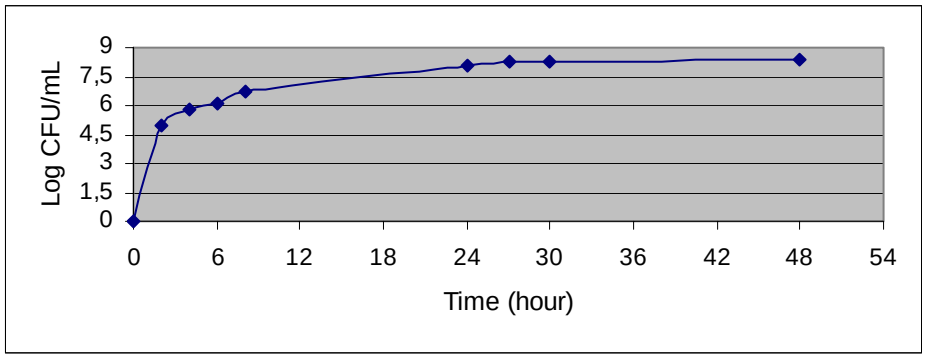


Figure 3.4. Growth of *Btk* 81 in previously optimized YSM.

Table 3.3. Sporulation frequency of *Btk*81 previously optimized YSM.

Incubation time (h)	Sporulation frequency (S/V)
6	$2,3x10^{-4}$
9	$1,8x10^{-3}$
12	$2,1x10^{-3}$
16	$1,0x10^{-2}$
24	$2,6x10^{-2}$
30	$4,0x10^{-2}$
36	$1,2x10^{-1}$
48	$2,4x10^{-1}$
72	1.0

The carbon sources tested in the present study were glucose and sucrose. In order to compare different C:N ratios (1:1, 2:1, 4:1, 8:1, 10:1 20:1 and 30:1) in the same set of experiments, parallel cultures of *Btk* 81 were run for 72 h and cell growth, sporulation and toxin protein profile of *Btk* 81 were determined for each. Firstly, glucose was incorporated into YSM medium at various C:N ratios, then sucrose was checked for the same ratios and the results were compared.

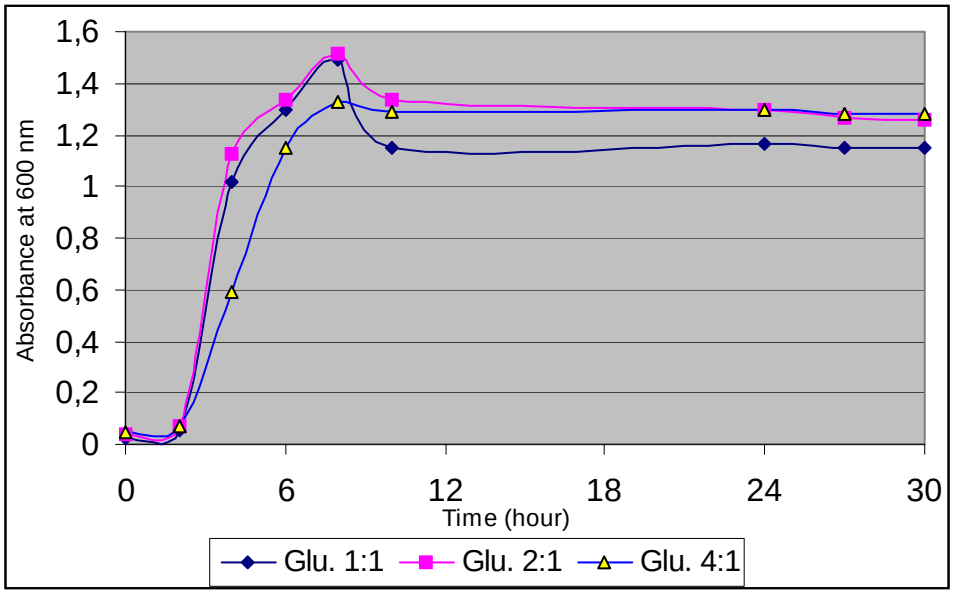
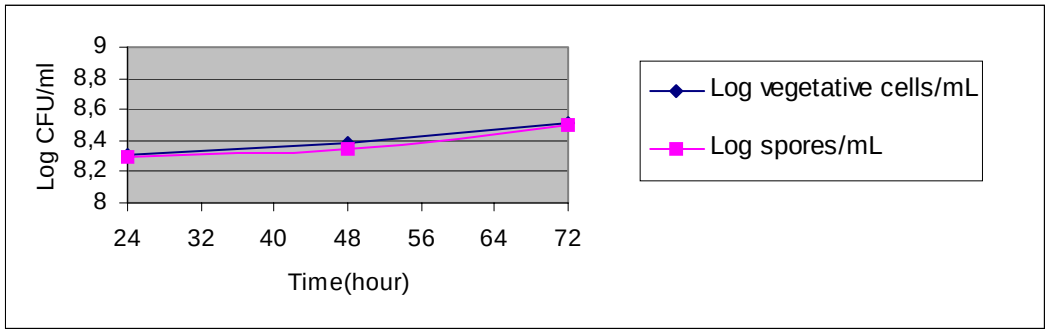


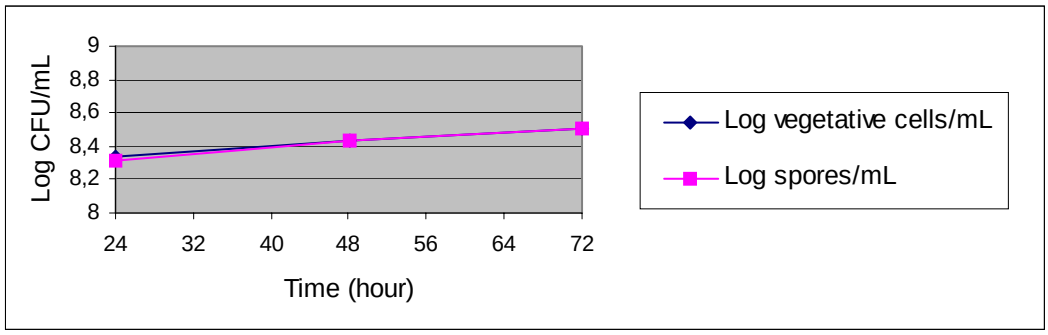
Figure 3.5 Growth of *Btk* 81 in YSM with glucose at three different carbon to nitrogen ratios.

İçgen *et al.*, (2002) had obtained the highest cell densities and lowest sporulation frequencies in YSM medium containing glucose. Toxin yields on this sugar was markedly less than those obtained on sucrose which was a not good carbon source for growth, but supported the synthesis of large amount of crystal protein as well as high sporulation frequencies. In present study, we tried to obtain high cell density as well as a high sporulation frequency with high toxin yield by varying C:N ratio on these processes. Figure 3.5 shows the growth obtained in YSM with C:N ratios of 1:1, 2:1 and 4:1, respectively.

a)



b)



c)

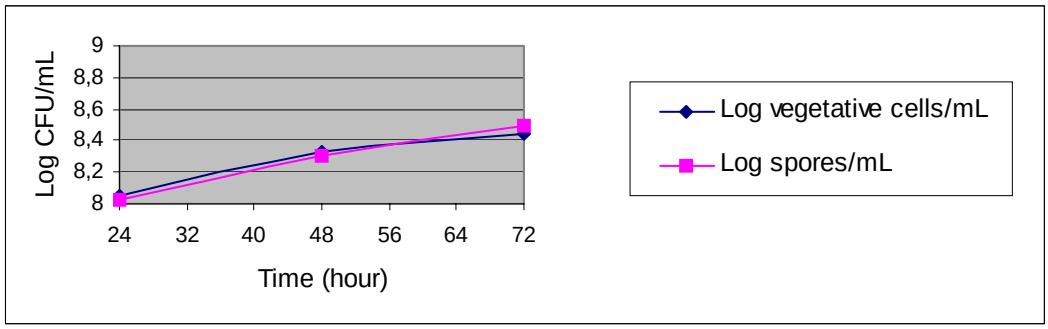
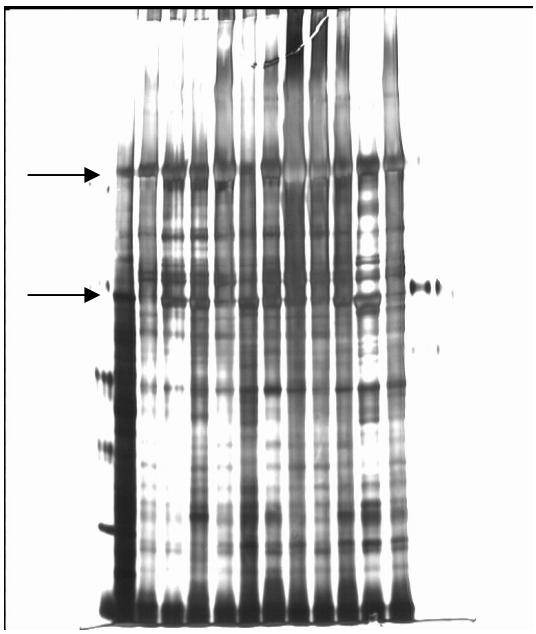


Figure 3.6. Effects of low C:N ratios [1:1(a), 2:1(b) and 4:1(c)] on growth and sporulation in YSM containing glucose.



M 1 2 3 4 5 6 7 8 9 10 11 12

M- Marker
1-1:1 24th h YSM
2-1:1 48th h YSM
3-1:1 72th h YSM
4-2:1 24th h YSM
5-2:1 48th h YSM
6-2:1 72th h YSM
7-4:1 24th h YSM
8-4:1 48th h YSM
9-4:1 72th h YSM
10-8:1 24th h YSM
11-8:1 48th h YSM
12-8:1 72th h YSM

Figure 3.7. Effect of low C:N ratio on the production of crystal toxins by Btk81 in YSM containing glucose.

When glucose was used at 1:1, 2:1, 4:1 and 8:1 C:N ratios, these conditions seemed to trigger a very high portion of vegetative cells to sporulate. These, ratios could be prefered for further crystal protein production trials in view of the final cell and spore concentrations compared (e.g. $4{,}6x10^8$ and $3{,}6x10^8$, respectively for C:N ratio 2:1). These values also indicated that sporulation frequency (S/V) approached to 1. Thus, full-sporulation seemed to be achieved under this condition. (Figure3.6).

Arcas *et al.*, (1987) had studied the effects of increasing concentration of the nutrients in glucose-yeast extract medium on growth, sporulation and endotoxin formation in batch cultures of *Btk* HD-1. They found that spore counts were increased from $1.08x10^{12}$ to $7,36x10^{12}$ spores/mL and toxin level from 1,05 mg/mL to 6.85 mg/mL, when the concentration of glucose was increased from 8 to 56 g/L, with the corresponding increase in the rest of medium components. Based on their approach, we grown *Btk* 81 at C:N ratios higher than 4:1 as well. However, when glucose was in excess (C:N of 10:1, 20:1 and 30:1) the growth was significantly inhibited and no 135 kDa and 65kDa *Btk* specific bands could not be observed in SDS-Polyacrylamide gel electrophoresis (Figure3.8, 3.9, 3.10).

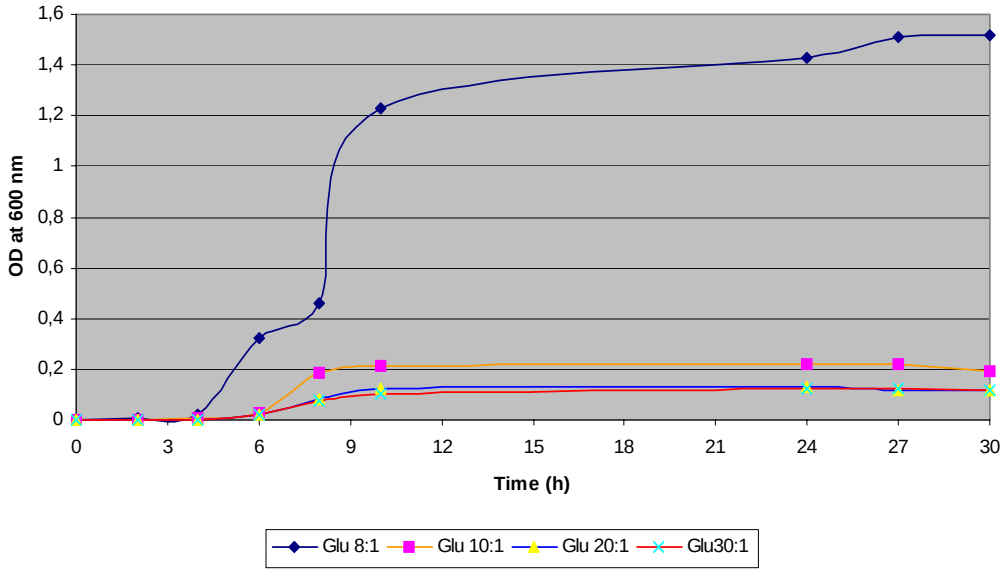
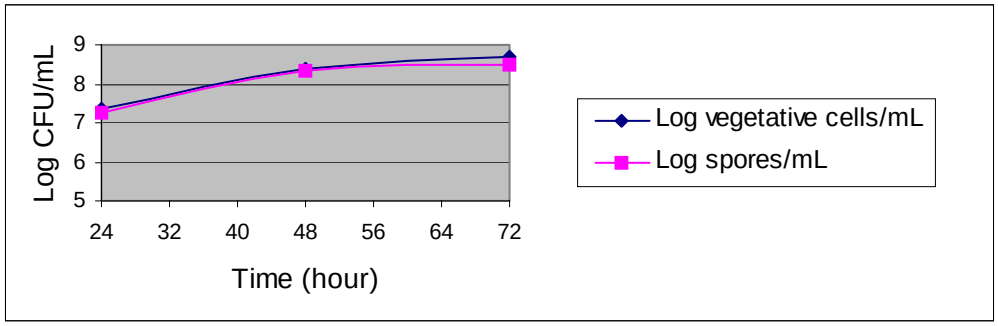
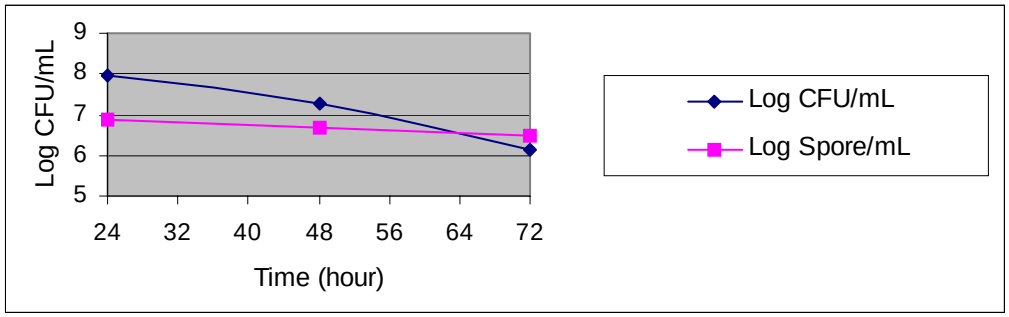


Figure 3.8. Effects of high C:N ratios on the growth of *Btk* 81 in YSM containing glucose.

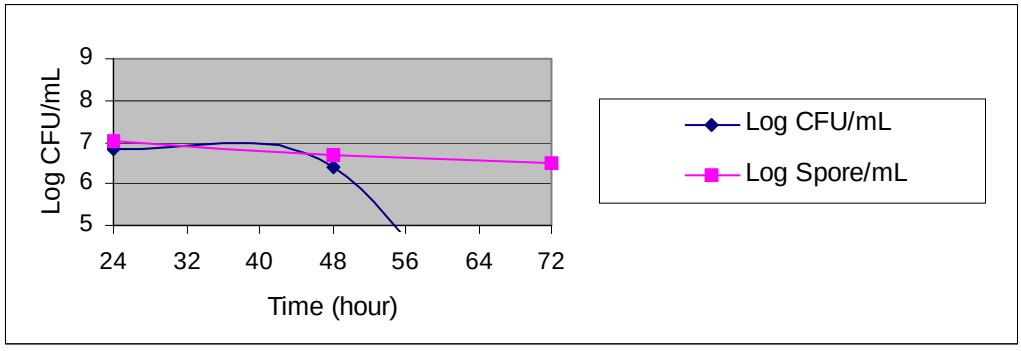
a)


Log CFU/mL
9
8
7
6
5
24
32
40
48
56
64
72
Time (hour)
Log vegetative cells/mL
Log spores/mL

b)


Log CFU/mL
9
8
7
6
5
24
32
40
48
56
64
72
Time (hour)
Log CFU/mL
Log Spore/mL

c)


Log CFU/mL
9
8
7
6
5
24
32
40
48
56
64
72
Time (hour)
Log CFU/mL
Log Spore/mL

d)

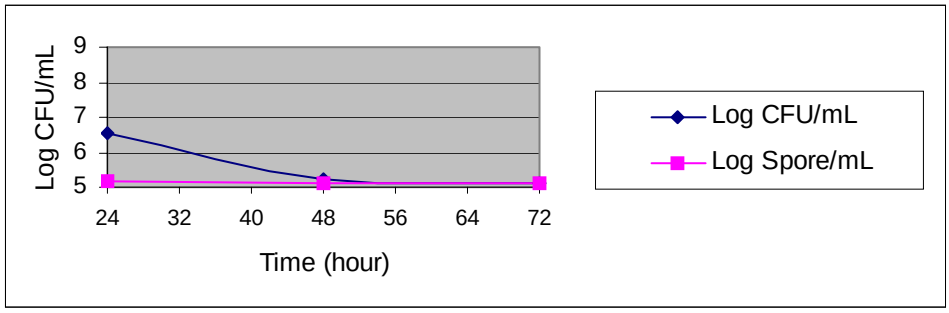
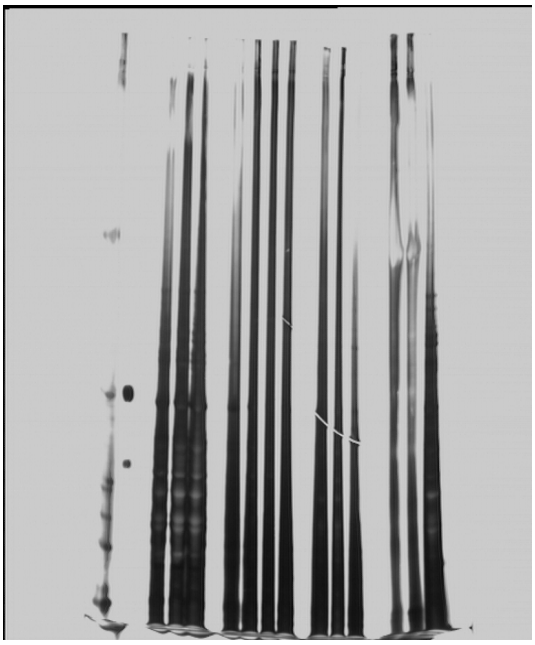


Figure 3.9. Effects of high C:N ratios [8:1(a), 10:1(b), 20:1(c) and 30:1(d)] on growth and sporulation in YSM containing glucose.



M 123 4 5 67 8 910 11 12 13

M- Marker
1- 8:1 24th h YSM
2- 8:1 48th h YSM
3- 8:1 72th h YSM
4- 10:1 24th h YSM
5- 10:1 48th h YSM
6- 10:1 72th h YSM
7- 10:1 72th h YSM
8- 20:1 24th h YSM
9-20:1 48th h YSM
10-20:1 72th h YSM
11-30:1 24th h YSM
12-30:1 48th h YSM
13-30:1 72th h YSM

Figure 3.10. Effect of high C:N ratios on the production of crystal toxins by *Btk* 81 in YSM containing glucose.

When sucrose was next tested at the same C:N ratios as was made for glucose, it showed different behaviors at low C:N ratios (1.1, 2:1, 4:1) (Figure 3.11 and 3.12). While cell growth, and sporulation seemed to increase until 72 nd hour in glucose media, no more increase in numbers of cells and spores was detected after 24th hour, in glucose media with C:N ratio ≥10:1 (Figure3.9) of but was decreased cell growth stopped at around 48th hour and death phase started. In spite of these, still 135 kDa and 65 kDa *Btk* 81 toxin bands were visualized by SDS-polyacrylamide gel electrophoresis.Yet, the crystal protein level seemed to be comparable to obtain glucose based low C:N ratios 1:1, 2:1, 4:1 and 8:1, respectively (Figure3.10).

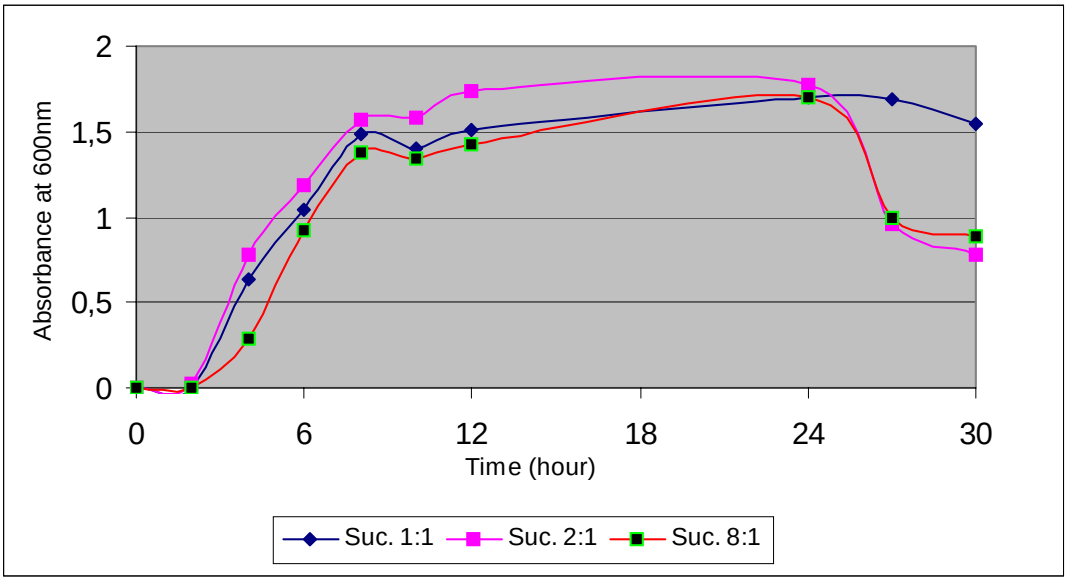
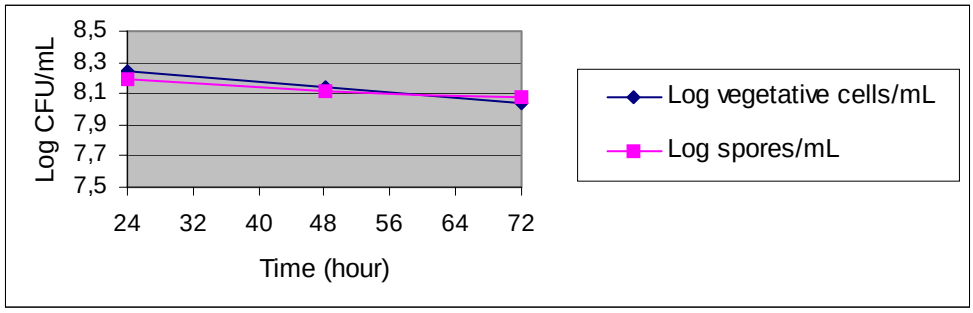
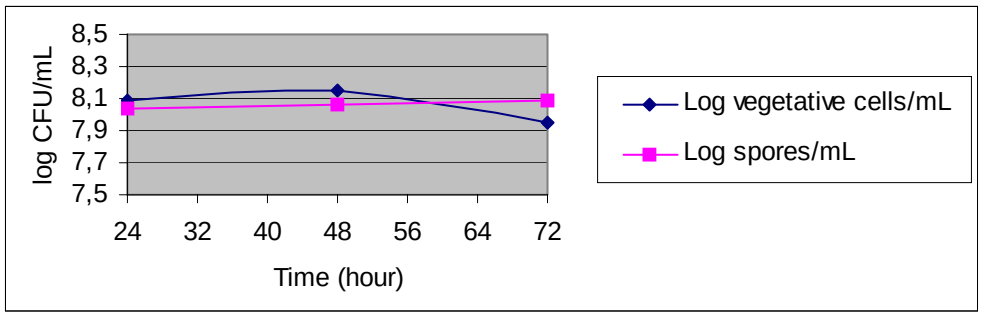


Figure 3.11. Effects of low C:N ratio on the growth of *Btk* 81 in YSM containing sucrose.

a)



b)



c)

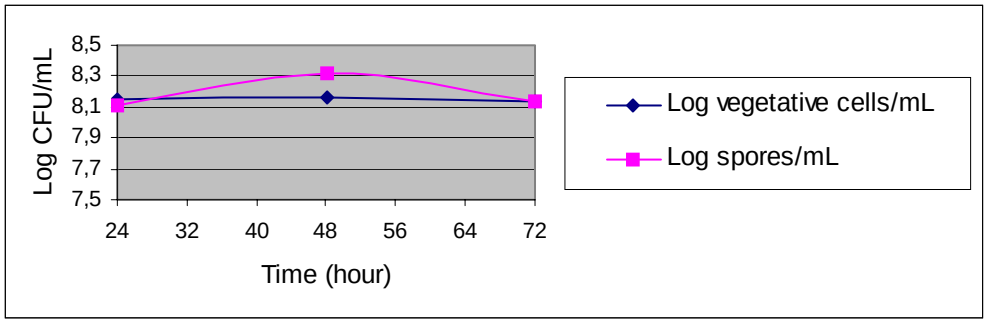
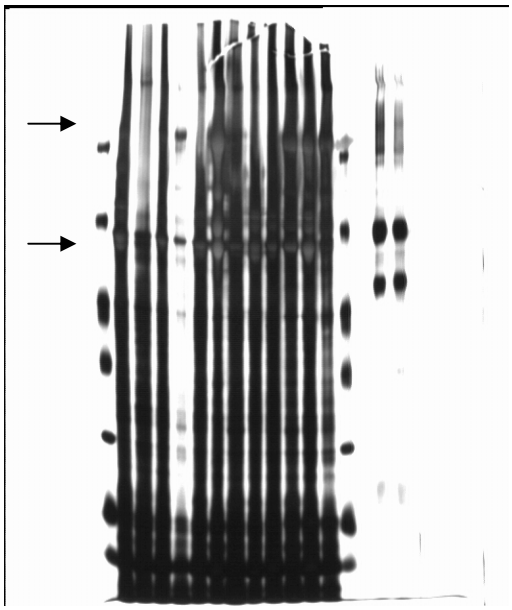


Figure 3.12. Effects of low C:N ratios [(1:1(a), 2:1(b) and 4:1(c)]on growth and sporulation in sucrose media for *Btk* 81.

M 123 456 789 10 11 12

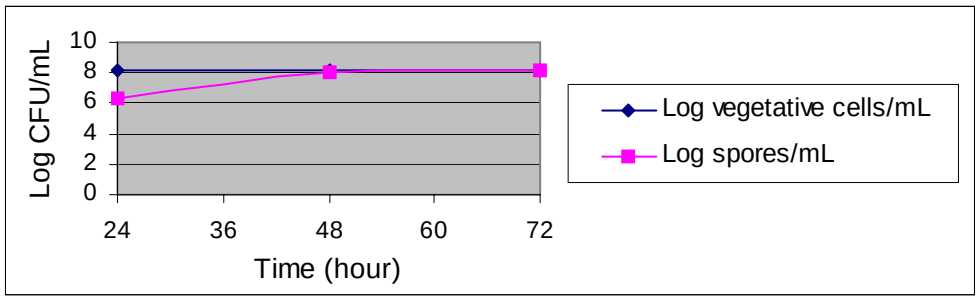


M- Marker
1- 24th h YSM
2- 48th h YSM
3- 72th h YSM
4- 1:1 24th h YSM
5- 1:1 48th h YSM
6- 1:1 72th h YSM
7- 2:1 24th h YSM
8- 2:1 48th h YSM
9- 2:1 72th h YSM
10- 4:1 24th h YSM
11- 4:1 48th h YSM
12- 4:1 72th h YSM

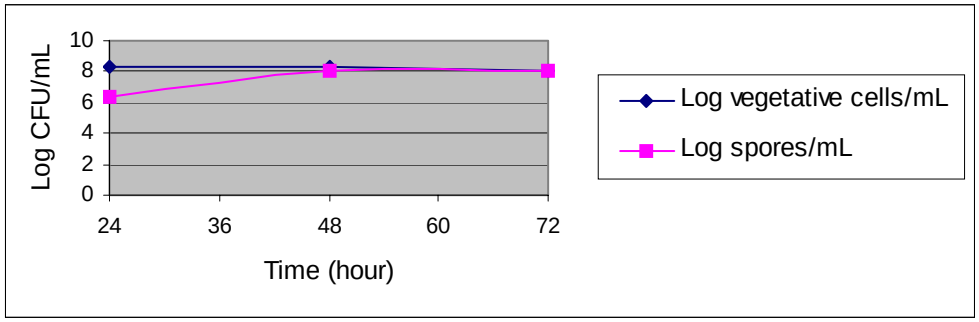
Figure 3.13. Effect of low C:N ratio on the production of crystal toxins of *Btk* 81 when the carbon source was sucrose.

At the range of 8:1 to 30:1 high C:N ratios of sucrose a decrease after 24th hour in the numbers of vegetative cells and spores was not detected which was the case at low C:N ratios with this sugar (Figure 3.13 and 3.14).In addition, the expression levels of the 135 kDa and 65 kDa crystals have increased. When all the quatitative toxin data for both glucose and sucrose varying C:N ratios were compared, it was determined that the crystal protein concentrations had the highest value in sucrose based medium when C:N ratio was 10:1 (Figure 3.15, Table 3.4).

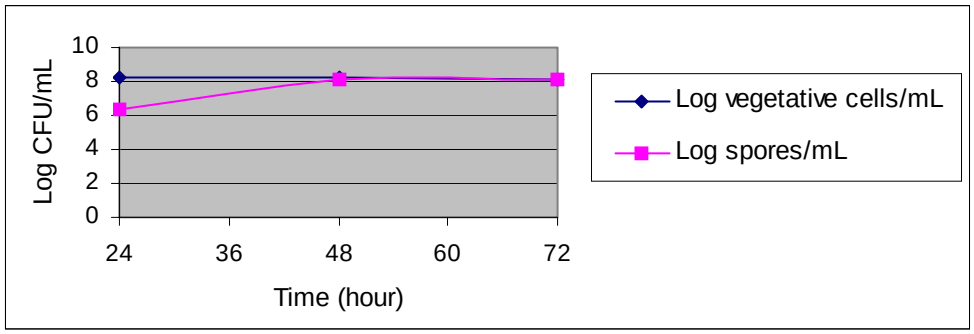
a)


Log CFU/mL
10
8
6
4
2
0
24
36
48
60
72
Time (hour)
Log vegetative cells/mL
Log spores/mL

b)


Log CFU/mL
10
8
6
4
2
0
24
36
48
60
72
Time (hour)
Log vegetative cells/mL
Log spores/mL

c)


Log CFU/mL
10
8
6
4
2
0
24
36
48
60
72
Time (hour)
Log vegetative cells/mL
Log spores/mL

d)

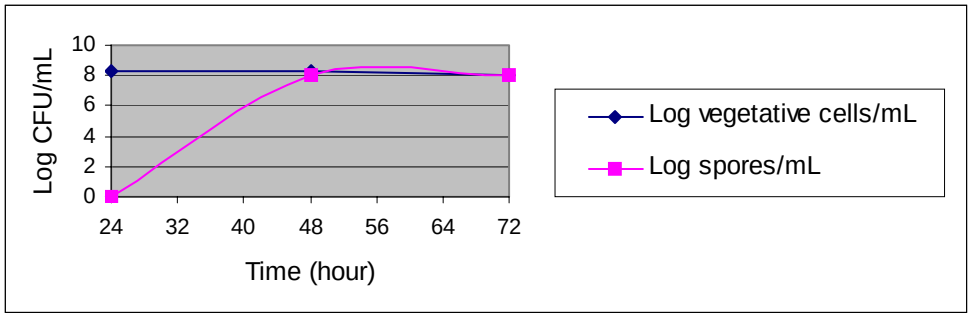
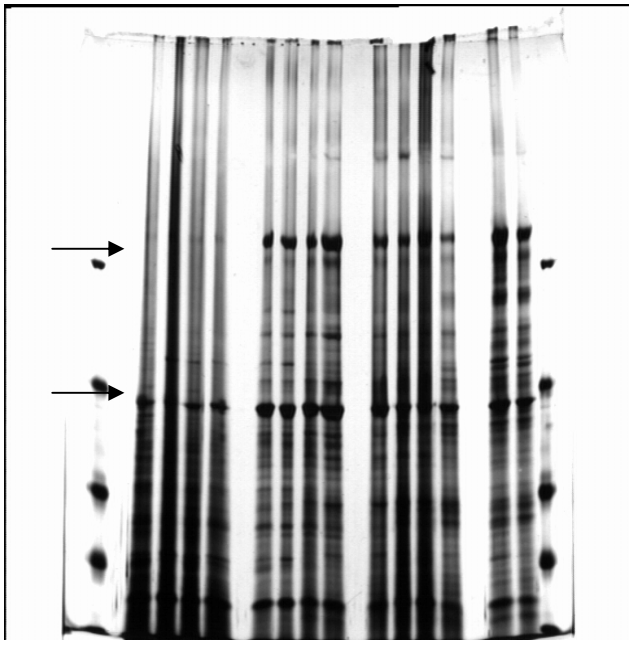


Figure 3.14. Effects of high C:N ratios [8:1(a), 10:1(b), 20:1(c) and 30:1(d)] on growth and sporulation in sucrose based YSM.



M 1 2 3 4 5 6 7 8 9 10 11 12 13 14

M- Marker
1-8:1 16th h YSM
2-8:1 24th h YSM
3-8:1 48th h YSM
4-8:1 72th h YSM
5-10:1 16th h YSM
6-10:1 24th h YSM
7-10:1 48th h YSM
8-10:1 72th h YSM
9-20:1 16th h YSM
10-20:1 24th h YSM
11-20:1 48th h YSM
12-20:1 72th h YSM
13-30:1 24th h YSM
14-30:1 48th h YSM
15-M

Figure 3.15. Effects of high C:N ratio on crystal protein production in sucrose based YSM.

Table 3.4. Comparison of crystal protein concentrations in three sets of conditions with promising results.

Binary toxin concentration (mg/mL) [Cry1Aa / Cry2Aa]				
Time (h)	**YSM $_{GLUCOSE}$ C:N 1:1**	**C:N 2:1**	**C:N 4:1**	**C:N 8:1**
24	0,0017 / 0,0024	0,0031 / 0,0026	0,0026 / 0,0015	0,0027 / 0,0018
48	0,0025 / ND	0,0027 / 0,0016	0,0029 / 0,0016	0,0031 / 0,0028
72	0,0030 / 0,0026	0,0019 / 0,0017	0,0028 / 0,0012	0,0028 / ND
Time (h)	**YSM $_{SUCROSE}$ C:N 8:1**	**C:N 10:1**	**C:N 20:1**	**C:N 30:1**
24	ND / 0,0028	0,0034 / 0,0036	0,0028 / 0,0030	0,0029 / 0,0027
48	ND / ND	0,0038 / 0,0042	0,0032 / 0,026	0,0026 / 0,0025
72	ND / 0,0026	0,0045 / 0,0048	0,0024 / 0,0025	----
Time (h)	**YSM $_{SUCROSE}$ C:N 1:1**	**C:N 2:1**	**C:N 4:1**	
24	0,0021 / 0,0019	ND / 0,0016	0,0020 / 0,0021	
48	ND / 0,0016	ND / 0,0015	0.0018 / 0,0020	
72	0,0014 / 0,0015	ND / 0,0015	0,0017 / ND	

Kang *et al.,* (1992) have developed an intermittent fed batch culture and achieved a high cell density of 72,6 g dry weight/L and a spore concentration of $1,25x10^{10}$ spores/mL. However, the toxin yields result were not recorded in this report. Ross and Mignone (1993) described a fed-batch process with increased spores, but with a 10 fold reduction in toxicity. Therefore, it is not easy to investigate the factors of fermentation process to achieve both high cell density and high toxin productivity. It would be also misleading to consider toxin formation solely as an event accompanying sporulation. Inspite of the close association between the two events, toxin biosynthesis appears to have its own controls. Thus, further studies on fermentation optimization will be required.

3.3. Delta Endotoxin Production Efficiency of *Bacillus thuringiensis israelensis S*train ONR60 as the Function of Carbon to Nitrogen Ratio

In the present part of the study, regulation by C:N ratio of crystal protein biosynthesis was investigated for improving the production this protein by *Bacillus thuringiensis* subsp. *israelensis* ONR60 (*Bti* ONR60). *Bti* ONR60 the first larvacidal isolate of H14 serotype which shows high toxicity against mosquitoes and black flies. This strain synthesizes larvacidal proteins with the molecular sizes of 135 kDa, 128 kDa, 65 kDa and 27 kDa respectively.

The experiments were performed by using TBL medium (a peptone, glucose and salts medium). This medium was prefered since it was used in a number of patented works. (e.g. US Patent Reference, Patent number:4448885; US Patent Reference, Patent number: 4467036). TBL was reformulized at three different C:N ratios, 2:1, 4:1 and 8:1 respectively. Growth characteristic of *Bti* ONR60 in TBL was determined by viable cell counts (Figure 3.16).

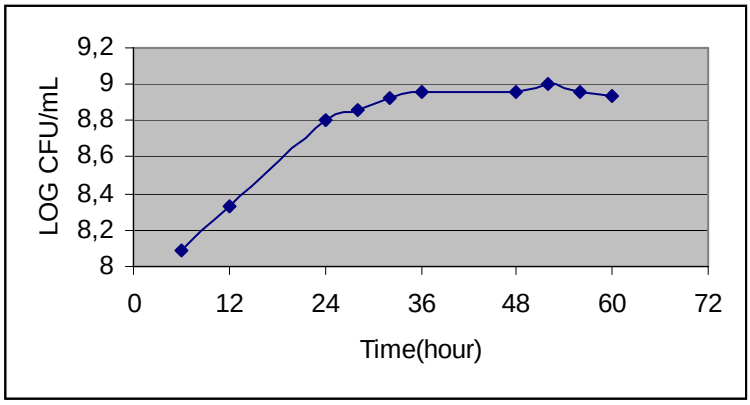


Figure 3.16. Growth of *Bti* ONR60 in TBL medium.

Although the studies related with *Bs* 2362 and *Btk* 81 were based on the increament of cell density and toxin production, in this part of the study the efficiency of *Bti* ONR60 was screened by bioassay analyses which measures the interaction between test larvae and the toxin being assayed. The most dramatic response of a larvae to microbial insecticide, and one that is the easiest to observe, is death. Thus, this approach provides the most accurate expression of killing power of the microbial insecticides.

TBL medium consists of glucose and peptone as the carbon and energy source. As the the first step protein profile of *Bti* ONR60 was visualized in original TBL medium and the results were compared to those obtained in YSM as our standard mediım for *Bt* subspecies. YSM was prepared in two forms. The first form had its original composition (AppendixA). However, the second one was re-designed medium with sucrose at about 1:1 C:N ratio. Thus, *Bti* ONR60 was

grown in three different media (TBL, $YSM_{ORIGINAL}$=$YSM_{GLUCOSE}$, $YSM_{SUCROSE}$). After SDS-polyacrylamide gel electrophoresis analysis, 10 mL of centrifuged (7000 rpm, 10 minutes) cultures was checked by bioassay for potency analysis (Figure 3.17, Table 3.6).

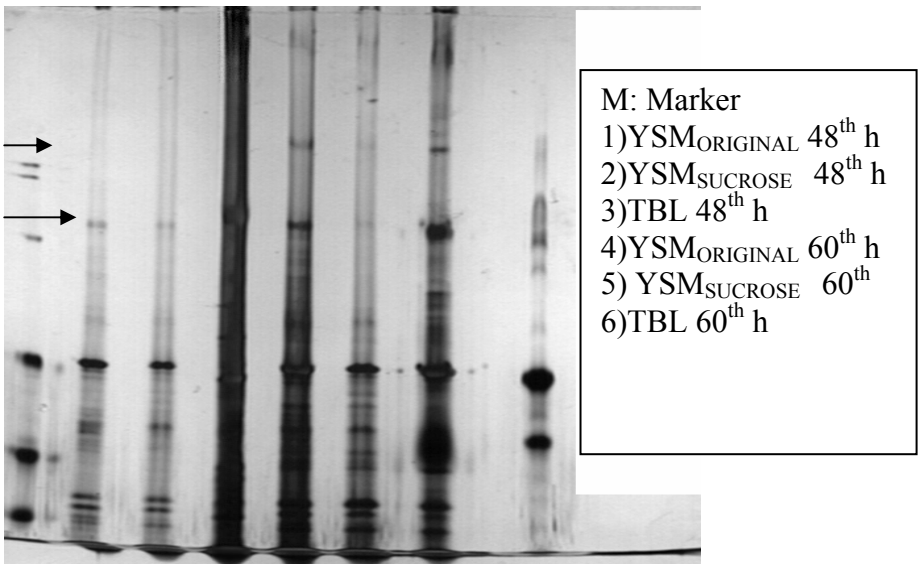


Figure 3.17. Protein profile of *Bti* ONR60 in three different media.

Table 3.5. Bioassay results of crystal toxin obtained in three different media.

Standart	Dosage (mg)	Initial number of larvae	Number of dead larvae	% Death rate
$YSM_{SUCROSE}$ 48	6,9	31	7	22,6
	6,6	18	10	55,5
	1,38	23	2	8,7
	0,69	27		0
$YSM_{SUCROSE}$ 60	12	11	6	54,5
$YSM_{SUCROSE}$ 72	7,7	24	24	100
$YSM_{GLUCOSE}$ 48	18,8	18	18	100
$YSM_{GLUCOSE}$ 60	9,3	17	16	94,1
	6,7	30	30	100
	1,34	26	15	57,7
	0,67	29	2	6,9
$YSM_{GLUCOSE}$ 72	13,2	17	16	94.1
	5,9	29	28	95,5
	1,18	27	3	11,1
	0,59	19	1	5,3
TBL 48	6,9	16	13	81,2
TBL 60	8	13	10	76,9
TBL 72	6,8	16	13	81,2

*Standart: Abott

When the Table 3.5 was examined as based on the dosage versus death rate and the dosages around 5 to 6 mg were compared, it can be deduced that the order of toxicity is $YSM_{GLUCOSE}$ 60 h > $YSM_{GLUCOSE}$ 72 h > TBL 48 h with death rates of 100%, 95.5% and 81.2%, respectively. Conclusively, $YSM_{GLUCOSE}$ medium was revealed to support the production of highest toxicity. Also, according to these data 60 hour appeared to be the most appropriate fermentation time for a toxicity product obtained in YSM.

Table 3.6. Results of another bioassay to include lower dosages of crystal toxin obtained in three different media.

Bti ONR60	Dosage (mg)	Initial number of larvae	Number of death larvae	% Death rate
$YSM_{SUCROSE}$ 48	6,12	20	2	10
	1,22	20		0
$YSM_{SUCROSE}$ 60	5,7	15	15	100
	3,6	24	13	54,2
	0,72	22	1	4,5
	0,36	19	1	5,2
$YSM_{GLUCOSE}$ 48	9,88	20	9	45
	1,97	20	3	15
$YSM_{GLUCOSE}$ 60	9,4	16	16	100
	4,6	25	25	100
	0,92	23	8	34,8
	0,46	23	2	8,7
TBL 48	4,6	20	2	10
	0,92	20	1	5
TBL 60	6,2	14	14	100
	1,6	30	30	100
	0,32	24	17	70,8
	0,16	20	10	50

However, when the applied doses were lowered next, TBLmedium appeared to the best as its much lowered doses (0,32 and 016 mg, respectively) gave rise to deaths of 70,8 and 50 %, respectively while an initial dose of 0,46mgprovided a larval death of only was 9 % when the medium was $YSM_{GLUCOSE}$. This finding is in good consistency with protein profiles obtained in SDS-polyacrylamide gel electrophoresis which revealed that much more toxin is produced in TBL (Figure 3.18).

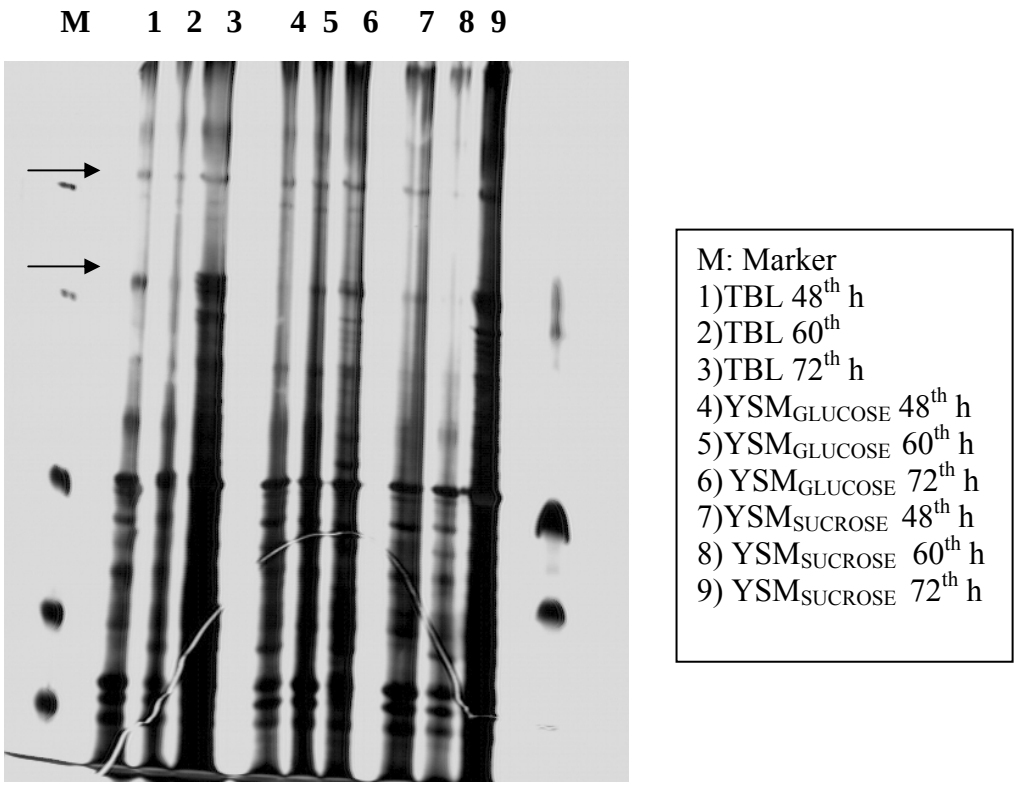


Figure 3.18. Protein Profile of *Bti* ONR60 in three different media and at 48th, 60th and 72th hour.

Some strains of *Bt* can utilize sugars other than glucose, such as fructose, sucrose, lactose etc. However, most of the industrially useful strains are not able to utilize high levels of sugars such as sucrose. Glucose has been used in most large scale Bt fermentations. Glucose concentration must be controlled in order to minimize the lag phase and maintain an exponential growth phase during fermentation. We tried to determine the critical levels of carbon to nitrogen ratio by re-designing TBL medium for four C:N ratios; 1:1, 2:1, 4:1, 8:1.

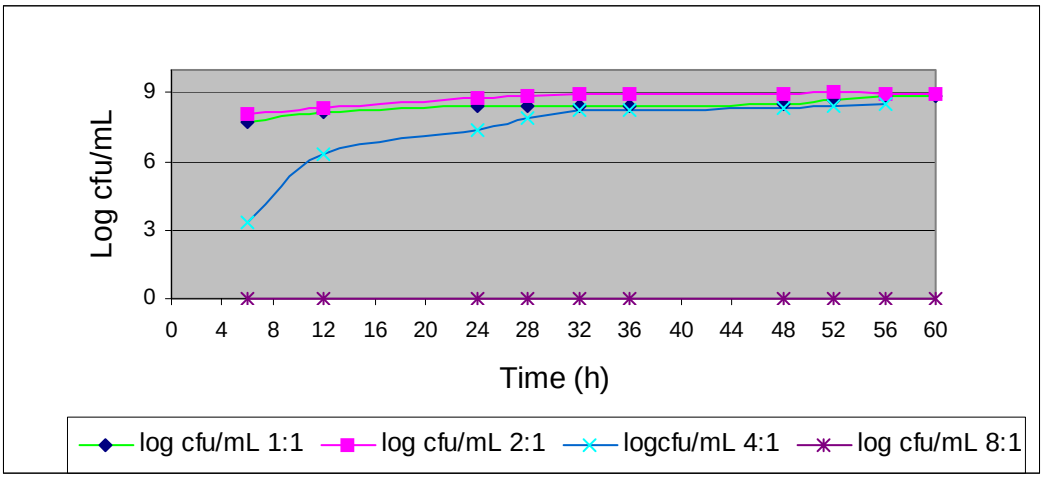


Figure 3.19. Growth of *Bti* ONR60 in TBL at four different C:N ratios.

As seen in Figure 3.19, the 8:1 C:N ratio did not support the growth. 2:1 C:N ratio has given the highest cell density. Although the highest cell concentration was maintained at 2:1 C:N ratio, productivity of the crystal protein contents of these cells were scanned by bioassay analysis (Table3.8).

Table 3.7. Comparison the effects of C:N of the medium TBL on the toxicity of the product.

Bti ONR60	Dosage (mg)	Number of initial larve	Number of death larvae	%death rate
TBL	2,4	20	20	100
	0,48	15	13	86,7
	0,24	23	6	26,1
TBL C:N 2:1	5,6	20	20	100
	2,3	20	20	100
	1,12	20	20	100
	0,56	21	20	95,2
	0,46	33	29	87,9
	0,23	30	14	46,7
TBL C:N 4:1	3,3	20	20	100
	0,66	20	20	100
	0,33	26	11	42,3
TBL C:N 8:1	5,7	20	20	100
	2,4	18	15	83,3
	1,14	21	15	71,4
	0,57	18	9	50
	0,48	21	5	23,8
	0,24	16		0
Standart				
TBL	7	20	20	100
	3,1	22	12	54,5
	1,4	21	17	80,9
	0,7	16	14	80,5
	0,62	28	1	3,6
	0,31	23		0
TBL C:N 2:1	2,7	20	20	100
	0,54	26	4	15,4
	0,27	20	1	5
TBL C:N 4:1	3,1	20	20	100
	0,62	22	19	86,4
	0,31	20	14	70
TBL C:N 8:1	2	25	2	8
	0,4	25		0
	0,2	25		0
IPS82	4,1	25	25	100

As seen in Table 3.7, *Bti* ONR60 grown in TBL at C:N ratios 2:1 and 4:1 gave given the high death rates even at low dosage applications For example, when Bti sample obtained from the culture grown at 2:1 C:N ratio applied at a dose of only 0,56 mg,Likewise, 066 mg of *Bti* sample 95 % death was recorded. Similarly, 0,66 mg of *Bti* sample obtained from the culture grown at 4:1 C:N ratio caused 100% death. These rates seemed to be comparable to standart which was Abott. On the other hand the bioassay results also show that 8:1 C:N ratio was poorly toxic to the larvae. In view of our results, TBL medium designed with 2:1 C:N ratio was chosen as the best for further steps. In addition, running time of the culture determined as 60 hours as was also determined in the previous experiment.

Traditional fermentation optimization studies involve optimization of nutrient requirement and as well as process parameters. However, despite the abundance of information on details of bacterial metabolism, there has been little quantitaive information regarding the carbon and nitrogen source utilization characteristics of *Bt.* Thus, we performed quntitative analysis to determine the amount of carbon and nitrogen consumption in the course of fermentation period. Total glucose was measured by the anthrone-sulfuric acid reaction. Nitrogen consumption was tested by Nitrogen (total) Cell Test kit. Results are tabulated in Table 3.8.

Table 3.8. Carbon-nitrogen utilization behavior of *Bti* ONR60 against time

Time (h)	Glucose (g/L)	Nitrogen (g/L)
0	5,614	ND
6	3,858	4,22
12	2,056	2,984
24	1,803	1,515
30	0,561	0,262
36	0,205	0
48	0,18	0
54	0,162	0
60	0,122	0

ND: Not determined (out of detectable range).

Luthy *et al.* (1982) demonstrated in *Bt* that glucose is assimilated through Embden Meyerhof pathway to produce acetate. Subsequently, the tricarboxylic acid cycle was found to be the major pathway for acetate assimilation and the provision of energy for growth and sporulation. Acetate which is accumulated during the exponential phase is then utilized in the early sporulating phase. In *Bacillus* spp. acetate assimilation starts while there is still a low level of glucose. Thus, it is important to know whether a fixed low level of glucose is the only factor that triggers acetate assimilation. It is equally important to know the critical level of acetate that has become inhibitory to growth.

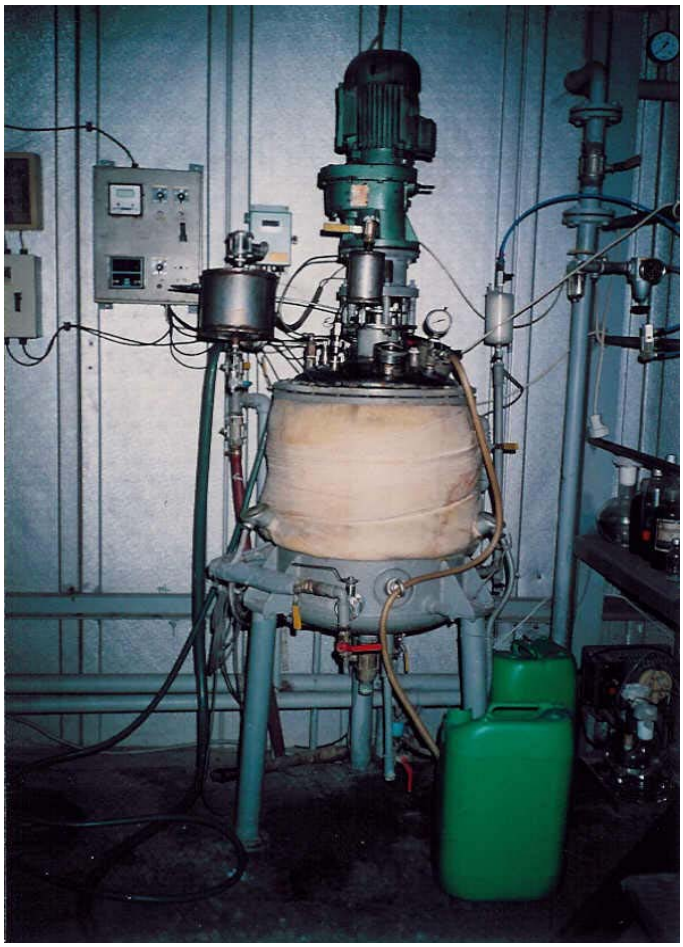
In some cases, limited glucose does not limit cell growth as long as there is a protein source in the culture (Chang, 1993). Generally, protein is utilized as carbon source when glucose was depleted. Bt sporulation is known to be initiated by the limitation of certain nutrients. Sakharova *et al.* (1984) investigated the

limitation of the following compounds for *Bt* subsp. strain galleriae: Glucose, yeast extract, phosphate, Mg^{2+} and K^{+}.

In the present part of the study, we determined the optimal C:N ratio for maximal toxicity prior to scaled-up studies. In the next section, this condition was applied to a 30 liter batch type fermentor.

3.4. Pilot Scale Production Trial with *B. thuringiensis* ONR60 in a 30L Batch Type Fermentor

As the last step of this study, the pre-determined optimal conditions were applied to a 30L batch type fermentor for toxin production by using *Bti* ONR60 (Figure 3.20).



Optimal conditions:

Medium: TBL with 2:1 C:N ratio

Running temperature:30^{o}C

Running period: 60 hours

Rotor frequency: 200 rpm

Figure 3.20. Pilot scale 30L batch type fermentor.

Fermentation studies were started by preparaing inoculum. Sterilization of the system was made by steam sterilization, performed two times at 121°C for 1 hour. Presented in Figure 3.21 is a flow scheme summarizing all operations including upstream and downstream processes.

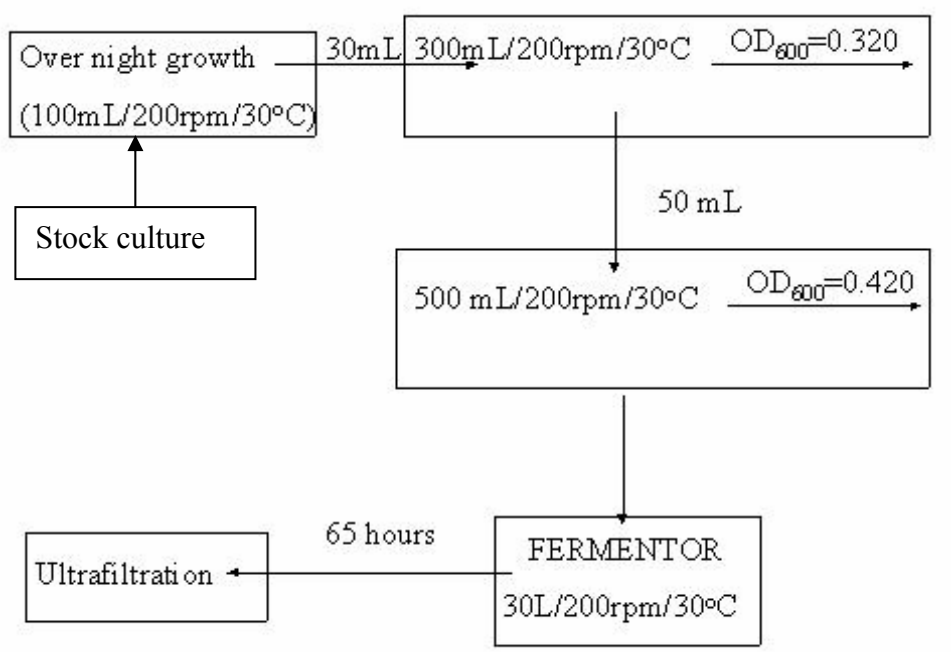


Figure 3.21. Schematic description of pilot scale fermentation studies.

As shown in Figure 3.21, *Bti* ONR60 cells were subcultured two times for inoculum preparation. In order to catch the cells at the exponential phase, growth was monitored spectrophotometrically in each subcultivation. 500 mL of *Bti* ONR60 culture was loaded into fermentor as the inoculum. In the course of

fermentation, cell growth, pH alterations and sporulation were analysed by taking aliquots at intervals.

Table 3.9. Changes in optical density and pH of the culture.

Time (h)	Absorbance at 600 nm	pH
18	1,365	6,81
24	DF:2 1,081	6,93
40	DF:2 1,354	7,13
48	DF:2 1,374	7,26
64	DF:2 1,298	7,08

DF: Dilution factor.

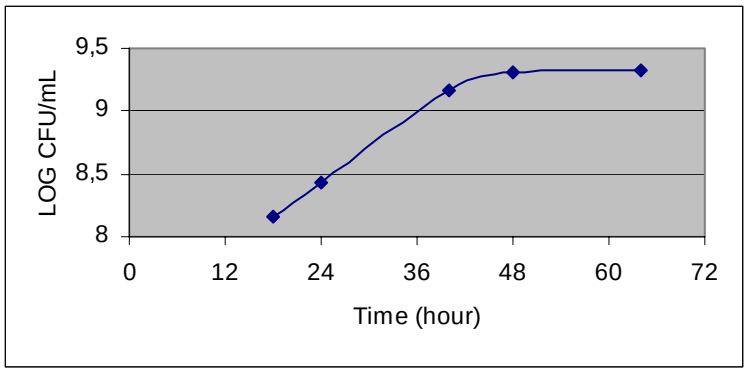


Figure 3.22. Growth of *Bti* ONR60 in the 30 L fermentor.

pH of the cell culture did not change drastically in the course of the operation (Table 3.9). Aeration rate of the fermentor was set to 0,75 v/v/m (0,75 volumes of air per volume culture suspension per minute). *Bti* ONR60 produces high concentrations of organic acids as a result of carbohydrate metabolism. In the case of low aeration rate, pH of the medium can drop below 6,5. When the pH was monitored in 200 ml culture in the batch flask, we observed a different picture (Figure 3.22) in that pH dropped below 6.5 and the culture was rather alkaline in aged cultures. In aerated fermentor, on the other hand, the air pumped possibly aided in removal of ammonium accumulated in culture, thus avoiding a rise in pH.

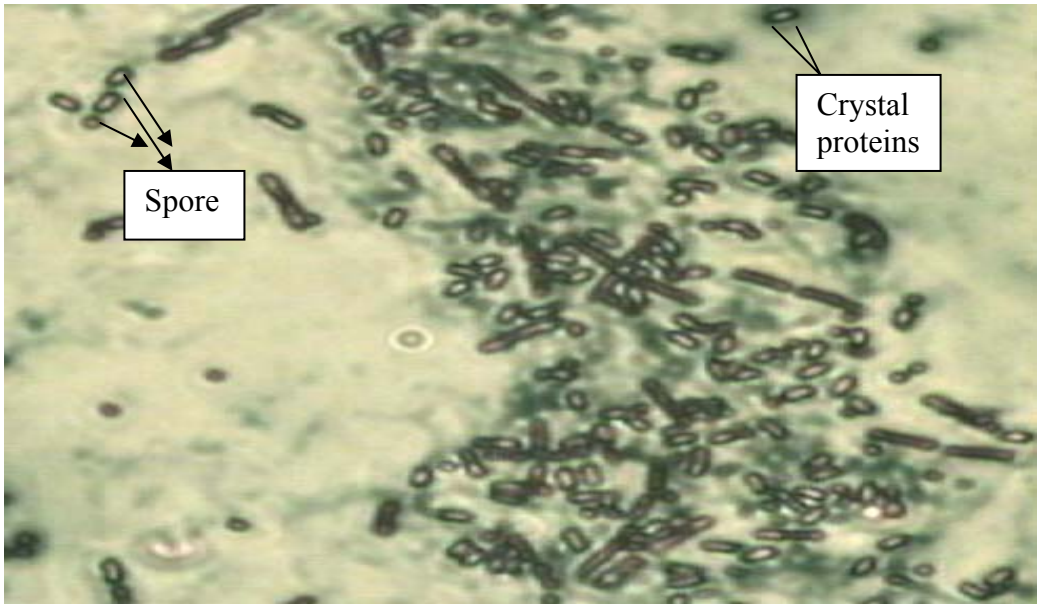
Table 3.10. pH alteration of culture when the experiment in 200 mL TBL medium

Time (h)	pH
0	7,22
3	7,02
6	6,52
9	6,3
12	6,61
24	7,84
27	7,51
30	7,7
33	7,85
36	7,96
48	8,33
51	8,26
54	8,37
57	8,49
60	8,53

When we compared the final cell densities reached in flask experiments and fermentor studies, we did not observe big difference. The cell concentration increased just 3 folds in the case of fermentor (Figure 3.16 and Figure 3.22).

Sporulation was monitored microscopically by taking aliquots at 6hours intervals. The spore and crystal protein content of the sample was visualized by staining the fixed smears with Comassie blue. Released spores with almost complete lysis of vegetative cells were detected at around 24^{th} hour of fermentation (Figure 3.23).

A



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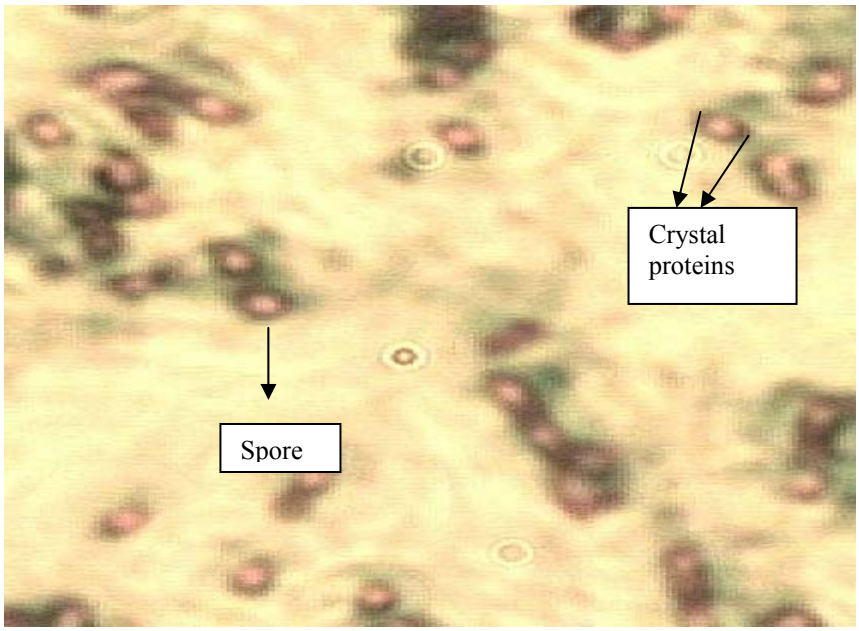

Figure3.23. Micrographs of released spores and crystals of *Bti* ONR6*0* with 40X (A) and 60X (B) magnification.

Bt sporulation is known to be initiated by the limitation of certain nutrients. Also dissolved oxygen (DO) levels lower than 10% were found to negatively effect sporulation and toxin production (Chang, 2000). *Bt* requires high oxygen uptake and generates large amount of heat during vegetative growth. Zamola and Kajfez (1977) developed and patented a novel intermittent use of aeration in *Bt* fermenatation. The authors found that interrupting aeration after 12-14 hours of growth prevented the lysis of the cells. The cell suspension was held without aeration for 3-6 hours and then returned to normal aeration. Thus, period with no aeration prevented sporulation, but allowed cell growth. The return of aeration allowed sporulation to proceed. As a result, when the suspension was harvested after 35-40 hours, sporulation and lysis were normal and essentially completed, with increased yields of toxin Goldberg *et. al.* (1980) also described a continuous fermentation and reported high growth rate and high spore yield. The aeration rate in their fermentors was 3-4 v/v/m. Thus, in the evaluation of commercial fermentation systems for *Bt* it is critical to make sure that large scale cultivation processes have adequate oxygen transfer and cooling capacity. Fed batch capability of *Bt* production fermentor i might be very useful to achieve high productivity.

Aeration also seemed to effect carbon and nitrogen consumption chracteristics of *Bti* ONR60 (Table3.11).

Table 3.11. Carbon and nitrogen utilization of *Bti* ONR60 against time in 30L batch fermentor.

Time (h)	Glucose(g/L)	Nitrogen g/L
24	0,052	0,962
48	0,0498	NT
64	0,0443	NT

NT: Not detected.

When we compared the carbon and nitrogen consumption caharacteristics of *Bti* ONR60, its consumption rate seemed to be higher in fermentor than that of 200 mL flask culture (Table 3.8 and Table 3.11).

After 64 hours of fermentation, product recovery was performed by ultracentrifugation. The centrifugation yielded a thick creamy product with a volume of about 800 mL. The larvacidal activity of the product was checked by bioassay. Unfortunately, the toxicity was not satisfactory, being much below the level of that expected as based on the results of the laboratory scale studies.

Since the toxin production plays the ultimate role in bio-control, high productivity is the major goal for optimization of *Bt* fermentation. Further lab-scale and pilot scale work towards this goal will be needed for process optimization and computer-controlled fed-batch processes can be useful for high protein productivity. Therefore future studies will also be focused on development of an efficient fed-batch process.

CHAPTER 4

CONCLUSION

- The growth of *Bs* 2362 was the best in NYSM and fairly good in SYSM. However, its level in WYSM was below the levels in NYSM and SYSM.

- Soy flour based media -SYSM- gave the promising results in terms of cell yield, sporulation frequency and binary toxin production by *Bs* 2362.

- In order to compare different C:N ratios (1:1, 2:1, 4:1, 8:1, 10:1 20:1 and 30:1) in the same set of experiments, parallel cultures of *Btk* 81 were run for 72 h and cell growth, sporulation and toxin protein profile of *Btk* 81 were determined for each. When all the quatitative toxin data for both glucose and sucrose varying C:N ratios were compared, it was determined that the crystal protein concentrations had the highest value in sucrose based medium when C:N ratio was 10:1.

- TBL medium designed with 2:1 C:N ratio was chosen as the best for further steps. In addition, running time of the culture determined as 60 hours as was also determined in the previous experiment.

- The pre-determined optimal conditions were applied to a 30L batch type fermentor for toxin production by using *Bti*. Unfortunately, the toxicity was not satisfactory.

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APPENDIX A

CULTURE MEDIA COMPONENTS

1. mYSM (İçgen ***et al, 2002)***

Component	*Concentration* (g/L)
$CaCl_2$. $2H_2O$	0.08
K_2HPO_4	100mM
$MgSO_4$. $7H_2O$	0.2
$MnSO_4$. H_2O	0.05
$(NH_4)_2HPO_4$	2
Yeast extract	2
Sucrose	1

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

2. Youten's Synthetic Medium (Yousten A.A., and Roegff 1969)

Component	*Concentration* (g/L)
$CaCl_2 . 2H_2O$	0.08
K_2HPO_4	0.5
$MgSO_4 . 7H_2O$	0.02
$MnSO_4 . H_2O$	0.05
$(NH_4)_2SO_4$	2
Yeast extract	2
Glucose	1

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

3. Nutrient Agar

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.

4. **Nutrient Broth Yeast Extract Sporulation Medium (Myers and Yousten, 1980).**

Component	*Concentration* (g/L)
Nutrient broth	8.0
Yeast extract	0.5
$MgCl_2.6H_2O$	0,2
$MnCl_2.4H_2O$	10.0mg
$CaCl_2.24H_2O$	0.1

The medium was sterilized at 121°C for 15 min.

5. **Whey Yeast Extract Sporulation Medium**

Component	*Concentration* (g/L)
Whey	8.0
Yeast extract	0
$MgCl_2.6H_2O$	0,2
$MnCl_2.4H_2O$	10.0mg
$CaCl_2.24H_2O$	0.1

The medium was sterilized at 121°C for 15 min.

6. Soy Powder Yeast Extract Sporulation Medium

Component	*Concentration* (g/L)
Soy powder	8.0
Yeast extract	0
$MgCl_2.6H_2O$	0,2
$MnCl_2.4H_2O$	10.0mg
$CaCl_2.24H_2O$	0.1

The medium was sterilized at 121°C for 15 min

7. TBL

Component	*Concentration* (g/L)
K_2HPO_4	4.35
KH_2PO_4	3.4
Salt solution	5 mL
$CaCl_2$ solution	5 mL
Bacto Peptone	7.5
Glucose	1.0

Salt solution

Component	*Concentration* (g/100mL)
$MgSO_4.7H_2O$	2.46
$MnSO_4.\ H_2O$	0.04
$Zn\ SO_4.\ 7H_2O$	0.28
$Fe\ SO_4.\ 7H_2O$	0.40

$CaCl_2.2\ H_2O$ solution

Component	*Concentration* (g/100mL)
$CaCl_2.2\ H_2O$	3.66

The pH was adjusted to 7.3 and the medium, except salt solution and $CaCl_2.2\ H_2O$ solution, was sterilized at 121°C for 15 min. Salt solution and $CaCl_2.2\ H_2O$ solution sterilized by filter sterilization.

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.

Running Buffer

30 g Trizma base

144 g Glycine

5 g SDS

Volume was made up to 5 L with distilled water.

4. 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

5. 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	Sigma
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