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MEDIUM DEVELOPMENT FOR PRODUCTION OF BACILLUS THURINGIENSIS BASED BIOPESTICIDES

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MEDIUM DEVELOPMENT FOR PRODUCTION OF *BACILLUS THURINGIENSIS* BASED BIOPESTICIDES

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

MEDIUM DEVELOPMENT FOR PRODUCTION OF *BACILLUS THURINGIENSIS* BASED BIOPESTICIDES

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The insect pathogen *Bacillus thuringiensis* (*Bt*) holds great promise as an effective and friendly way for management of the pests with safety for nontarget animals and humans. However, high capital investment due to high production and formulation cost of commercial *Bt* preparations has caused prohibitive effect on companies. The present study mainly aimed at developing a low cost medium that supports the growth of different *Bt* strains and their specific bioinsecticidal δ-endotoxins (crystal proteins).

A comparison was made between the representative members of three different subspecies of *Bt* to observe toxin yields in response to certain nutritional conditions. Three different *Bt* subspecies were *Bt kurstaki* (strain 81), *Bt israelensis* (strain HD500) and *Bt tenebrionis* (strain 3203), producing lepidoptera- and diptera-specific Cry1 and Cry2, diptera-specific Cry4Ba and Cry11Aa and coleoptera-specific Cry3Aa toxins, respectively. Studies were conducted to optimize glucose and inorganic phosphate

concentrations in standard DSM medium for the production of these *Bt*-based biopesticides. General suppression of toxin yields in high glucose medium (10 g/L) thought the generality of carbon catabolite regulation for biosynthesis of different types of toxins. Inorganic phosphate (Pi) level was important for Cry4Ba, Cry11Aa and Cry3Aa biosynthesis while Cry1 and Cry2 production was not responsive to high Pi. Wastewater sludge, fruit residues and broiler litter were next tested as cheap raw materials for *Bt*-based biopesticide production in batch cultures. Broiler litter seemed to be a much better substrate among all since some degree of production of each toxin was observed at almost every stage of fermentation. The processing of broiler litter was found to significantly improve toxin yields. The medium prepared from processed broiler litter was successfully used to cultivate all *Bt* stains and obtain bioinsecticidal proteins in high yields which were comparable or higher than those that can be obtained on standard semi-synthetic media.

Keywords: *Bacillus thuringiensis*, δ-endotoxin, Crystal Protein, fermentation, Low Cost Media

ÖZ

BACILLUS THURINGIENSIS BİYOPESTİSİTLERİNİN ÜRETİMİ İÇİN BESİYERİ GELİŞTİRİLMESİ

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Bir böcek patojeni olan *Bacillus thuringiensis* (*Bt*), zararlı böceklerle olan mücadelede hem etkin, hem de hedef olmayan hayvan ve insanlara karşı toksik etkiler göstermemesi nedeniyle güvenli ve çevre dostu bir seçenektir. Diğer yandan, üretim ve formülasyon maliyetinin yüksek olması, endüstriyel yatırım harcamalarını yükseltmekte, bu da yatırımcı firmalar üzerinde caydırıcı etki yapmaktadır. Şimdiki çalışmada, farklı *Bt* suşlarının üremelerini ve biyoinsektisidal δ-endotoksinler (kristal proteinler) üretmelerini destekleyecek düşük maliyetli bir besi ortamı geliştirilmesi hedeflenmiştir.

Öncelikle *Bt*'nin üç farklı ve önemli alt türünü temsil eden suşlar, bazı besinsel koşullara verdikleri yanıtlar bakımından karşılaştırılmışlardır. Kullanılan suşlar, *Bt kurstaki* (81 suşu), *Bt israelensis* (HD500 suşu) ve *Bt tenebrionis* (3203 suşu) alt türlerine bağlı olup, sırasıyla lepidoptera ve diptera'lara karşı etkin Cry1 and Cry2, sadece diptera'lara karşı etkin Cry4Ba ve Cry11Aa ve coleoptera'lara karşı etkin Cry3Aa toksinlerinin üreticisidirler.

Standard DSM besi ortamında bu suşlar tarafından ilgili toksinlerin üretimini destekleyecek glikoz ve inorganik fosfat konsantrasyonları optimize edilmeye çalışılmıştır. Suşların tümü için yüksek glikoz ortamının (10 g/L) toxin üretimini baskıladığı görülmüş, bu da karbon katabolit regülasyonun farklı δ-endotoksin tiplerinin biyosentezi genelinde geçerli olduğunu düşündürmüştür. İnorganik fosfat (Pi) seviyesinin, Cry4Ba, Cry11Aa ve Cry3Aa biyosentezini etkilediği, ancak Cry1 ve Cry2 biyosentezi üzerinde etkisi olmadığı bulunmuştur. Atık su çamuru, meyve posası ve tavuk dışkısı ucuz hammaddeler olarak kullanılmak suretiyle kesikli kültürlerde biyoinsektisid üretiminde kullanılabilecek ucuz bir besi ortamı geliştirilmesine çalışılmıştır. İncelenen tüm suşlarda toksin üretimini fermentasyonun hemen her aşamasında belli seviyelerde desteklemesi nedeniyle bunlar arasında en iyi substrat adayının tavuk dışkısı olduğu belirlenmiştir. Tavuk dışkısının ön işlemden geçirilmesi, toksin verimini önemli ölçüde arttırmıştır. Ön işlemden geçirilmiş tavuk dışkısı ile hazırlanmış besi ortamı, tüm *Bt* suşlarının kültivasyonu ve standart semisentetik besi ortamlarında elde edilebilecek seviyelerde ya da üstünde toxin üretimi için başarıyla kullanılmıştır.

Anahtar Kelimeler: *Bacillus thuringiensis*, δ-endotoksin, Kristal Protein, Fermantasyon, Ucuz Besiyeri

To My Family

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LIST OF ABBREVIATIONS

Bt	*: Bacillus thuringiensis*
Btt	*: Bacillus thuringiensis* subspecies *tenebrionis*
Bti	*: Bacillus thuringiensis* subspecies *israelensis*
Btk	*: Bacillus thuringiensis* subspecies *kurstaki*
ICP	: Insecticidal Crystal Protein
kDa	: Kilodalton
mDa	: MegaDalton
DSM	: Difco's Sporulation Medium
SDS-PAGE	: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
aa(s)	: Amino acid(s)
bp	: Base pairs
kb	: Kilobase
IUPAC	: International Union of Pure and Applied Chemistry
BGSC	: Bacillus Genetic Stock Center
NCBI	: National Center for Biotechnology Information
SEM	: Scanning Electron Microscope

CHAPTER 1

INTRODUCTION

1.1. *Bacillus thuringiensis*

According to the UN Food and Agricultural Organization, about 55% of the world's potential human food supply is lost to pests before (35%) or after (20%) harvest. The use of synthetic chemical insecticides has played a key role not only in the increase of agricultural productivity and protection of crops and forests, but also in the control of insect vectors of human diseases such as dengue haemorrhagic fever and malaria (Becker *et al*., 1993).

In the developed countries, crop losses are estimated to be in the range of 10–30%, while in developing nations they are estimated to be as high as 75% (Ohayo-Mitoko, 1997). Pesticide application has been promoted among farmers in developing countries to increase their productivity, translating into improved incomes and food provision for farm households. Agricultural intensification based on production technologies with chemical inputs including pesticides is in fact widely used as a strategy for poverty alleviation and food security among smallholder farmers (Pesticide Action Network UK, 2002). A significant increase in pesticide use has increased concerns about potentially adverse effects on human health and the environment, particularly in countries where regulations are not strictly implemented and farmers' knowledge of safe handling procedures is often inadequate (Snelder *et al*., 2007).

As hazards of conventional, broad acting pesticides are documented, researchers look for pesticides that are toxic only to the target pest, have less impact on other species, and have fewer environmental hazards (Frankenhuyzen, 1993). The experimental results agreed with farmers' affirmative response to questions about their suffering various symptoms of poisoning. Estimates of pesticide concentrations in watercourses exposed to drift suggest that aquatic species will suffer adverse effects up to at least 2.0m from field borders (Snelder *et al*., 2007).

The method alternative to insect pest control by the use of chemicals is the use of microbial insecticides (Steinhaus, 1956). Such insecticides consist of microorganisms or their by-products. Microbial insecticides are target-specific, thus, safe for human, non-targeted plants and animals as well as environmentally friendly for being biodegradable and non-accumulating. The most widely used microbial control agent is the bacterium *Bacillus thuringiensis* (*Bt*) (Lacey *et al*., 2001). The main reason for this is the production of parasporal crystal inclusions during the sporulation stage. These crystals, composed of delta-endotoxins or Cry-proteins, are the basis of its toxicity and specificity against various insect larvae (lepidoptera, diptera, and coleoptera) and nematodes (Barloy *et al*., 1998). They are produced at the time of sporulation and account for up to 30% of the total protein content of the bacterium (Lacey *et al*., 2001).

The other entomopathogenic microorganisms used for microbial control include baculoviruses, protozoa, fungi, nematodes and some bacteria such as *Bacillus popilliae, Brevibacillus laterosporus, Clostridium bifermentans, Penibacillus spp.* (Charles and Nielsen-LeRoux, 2000). *Bt* technology on certain aspects of environmental health, such as the potential for adverse affects on non-target insects, particularly on beneficial arthropods. However, there have been very few studies where the affects of the *Bt* technology on

soil microorganisms or soil ecology have been evaluated (Devare *et al*., 2004).

Bt was started to be used for insect pest control in the late 1920s and in the early 1930s when it was used against *Ostrinia nubulis*, the European corn borer (YieldGard® Corn Borer, 2007). *Bt* became available as a commercial insecticide in France as spore-based formulations called Sporeine to kill flour moths in 1938 (Weiser, 1986) and in the 1950s entered commercial use in the United States (Faust, 1974). Commercial production and worldwide use of *Bt* was well established by the early 1980s. In 1961, *Bt* subsp. *kurstaki* (*Btk*) was used as a biopesticide and *Btk*-based products for the control of susceptible lepidopteran pests in agriculture and forestry became predominant in markets (Lüthy *et al*., 1982). New markets were opened by the 1976 discovery of the *israelensis* subspecies, which is toxic to larval mosquitoes and black flies (Goldberg and Margalit, 1977) and the discovery of *Bt* subsp. *tenebrionis* which is toxic to several beetle species (Krieg *et al*., 1983).

Bt is a facultative anaerobic gram-positive bacterium present in soil, water and on plant surfaces. There are over then 12.145 strains of *Bt* in *Bt* bank at United States Department of Agriculture (USDA) (http://www.ars.usda.gov/IS/AR/archive/mar96/bt0396.htm). To date, over 12 thousand natural strains have been isolated from various geographical areas and from different sources, including grain dust, soil, death insects and plants. These isolates can be classified into about 30 serotypes based on biochemical properties, flagellar antigens and H-antigens (De Barjac and Franchon, 1990). However, this classification does not reflect the pathotype of the bacteria which is essentially defined by the delta endotoxins (δ-endotoxins) that make up the characteristic crystalline inclusions of the *B. thuringiensis* strains (Figure 1.1).

The crystals of *Bt* are mainly composed of insecticidal toxin proteins, with some strains producing a single toxin and others multiple toxins. The production of the toxin crystal is tightly coupled to sporulation; the mechanism of this co-ordination can largely be explained by the fact that most of the toxin genes are transcribed by the sporulation-specific σ^E- and/ or σ^K -containing RNA polymerase (Baum and Malvar, 1995). There are, however, exceptions with some toxin genes, e.g. Cry3A, being transcribed in the vegetative cell (Agaisse and Lereclus, 1994b). Despite transcription at this earlier stage the crystals themselves are only assembled during sporulation.

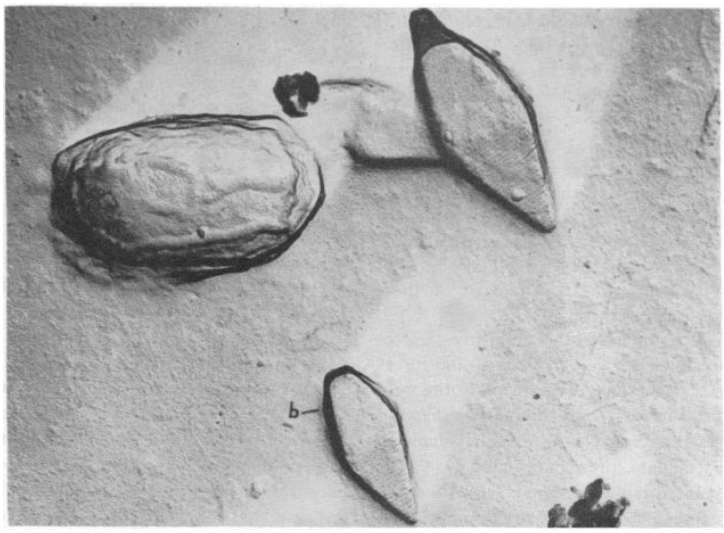


Figure 1. 1. Spore and crystals of *Bacillus thuringiensis Berliner* (Monro, 1959).

1.2. Cry Toxins

1.2.1. Diversity, Structure and Evolution of Cry Toxins

Bt Cry and Cyt toxins belong to a class of bacterial toxins known as pore-forming toxins (PFT) that are secreted as water-soluble proteins undergoing conformational changes in order to insert into, or to translocate across, cell membranes of their host. Cry proteins are defined as: a parasporal inclusion

protein from *Bt* that exhibits toxic effects to a target organism, or any protein that has obvious sequence similarity to a known Cry protein. Cyt toxins are included in this definition but it was agreed that proteins that are structurally related to Cyt toxins retain the mnemonic Cyt. Primary sequence identity among different gene sequences is the bases of the nomenclature of Cry and Cyt proteins. Additionally, other insecticidal proteins that are not related phylogenetically to the threedomain Cry family have been identified. Among these, are binary-like toxins and Mtx-like toxins related to *B*. *sphaericus* toxins, and parasporins produced by *Bt* (Crickmore *et al*., 1998).

As mentioned earlier, *Bt* produces δ-endotoxins during the sporulation phase of growth. The δ-endotoxins comprise two multigenic families, Cry and Cyt (cytolysins) (Bravo *et al*, 2007). Knowles and Ellar (1988) have pointed that crystal proteins can be grouped according to host range and classified under four major groups. In 1989, Hofte and Whiteley reviewed systematic nomenclature and classified crystal proteins into five major groups according to their insecticidal and molecular relationships (CryI, CryII, CryIII, CrylV and CryV, Cyt). The discovery of new strains having an activity spectrum that spans two or three insect orders (such as those producing CryIB protein that shows insecticidal activity against both Lepidoptera and Coleoptera) led the scientist to establish a new classification system based on amino acid sequence homology (Schnepf *et al*., 1998).Revised gene names used in this new classification system are listed in Table 1.1. New nomenclature has defined four hierarchical ranks. Romen numerals, which comes after the Cry or Cyt words, have been exchanged for Arabic numerals in the primary rank, it is followed by other three hierarchical rank consisting of numbers, capital letters, lower case letters and numbers (e.g. Cry23Aa1), depending on its place in a phylogenetic tree (Figure 1.2). Proteins with the same primary rank often affect the same order of insect; those with different secondary and tertiary ranks may have altered potency and targeting within an order. The quaternary rank was established to group “alleles” of genes coding for known

toxins that differ only slightly, either because of a few mutational changes or an imprecision in sequencing. The proteins less than 45 % sequence identity differ in primary rank (Cry1, Cry2, etc.) and 78 % and 95 % identity constitute the borders for secondary and tertiary rank, respectively (Crickmore *et al*., 1998). *Cyt* and *cry* genes do not share significant sequence homology; however, both types of toxins seem to work through pore formation that leads to cell lysis and irreversible damage of the insect midgut (Knowles *et al*., 1987; Knowles *et al*., 1989). While Cry toxins act via specific receptor recognition and binding (Gill *et al*., 1987), no specific receptors have been described for Cyt toxins, although they show specificity of action *in vivo* (Koni and Ella, 1994).

Table 1. 1. Known *cry* and *cyt* gene sequences with revised nomenclature assignments (Crickmore *et al*., 1998).

Revised gene name	Original gene or protein name	Accession no.	Coding region[a]	Reference
cryLAa1	*cryLA(a)*	M11250	527–4054	92
cryLAa2	*cryLA(a)*	M10917	153–>2955	98
cryLAa3	*cryLA(a)*	D00348	73–3600	99
cryLAa4	*cryLA(a)*	X13535	1–3528	62
cryLAa5	*cryLA(a)*	D17518	81–3608	113
cryLAa6	*cryLA(a)*	U43605	1–>1860	63
cryLAb1	*cryLA(b)*	M13898	142–3606	119
cryLAb2	*cryLA(b)*	M12661	155–3622	111
cryLAb3	*cryLA(b)*	M15271	156–3620	31
cryLAb4	*cryLA(b)*	D00117	163–3627	50
cryLAb5	*cryLA(b)*	X04698	141–3605	40
cryLAb6	*cryLA(b)*	M37263	73–3537	37
cryLAb7	*cryLA(b)*	X13233	1–3465	36
cryLAb8	*cryLA(b)*	M16463	157–3621	69
cryLAb9	*cryLA(b)*	X54939	73–3537	13
cryLAb10	*cryLA(b)*	A29125	—[b]	28
cryLAc1	*cryLA(c)*	M11068	388–3921	3
cryLAc2	*cryLA(c)*	M35524	239–3769	117
cryLAc3	*cryLA(c)*	X54159	339–>2192	18
cryLAc4	*cryLA(c)*	M73249	1–3534	84
cryLAc5	*cryLA(c)*	M73248	1–3531	83
cryLAc6	*cryLA(c)*	U43606	1–>1821	63
cryLAc7	*cryLA(c)*	U87793	976–4509	38
cryLAc8	*cryLA(c)*	U87397	153–3686	71
cryLAc9	*cryLA(c)*	U89872	388–3921	33
cryLAc10		AJ002514	388–3921	107
cryLAd1	*cryLA(c)*	M73250	1–3537	79
cryLAe1	*cryLA(e)*	M65252	81–3623	60
cryLAf1	*icp*	U82003	172–>2905	49
cryIBa1	*cryIB*	X06711	1–3684	10
cryIBa2		X95704	186–3869	105
cryIBb1	ET5	L32020	67–3753	25
cryIBc1	*cryIB(c)*	Z46442	141–3839	6
cryIBd1	*cryE1*	U70726		12
cryICa1	*cryIC*	X07518	47–3613	45
cryICa2	*cryIC*	X13620	241–>2711	88
cryICa3	*cryIC*	M73251	1–3570	79
cryICa4	*cryIC*	A27642	234–3800	114
cryICa5	*cryIC*	X96682	1–>2268	106
cryICa6	*cryIC*	X96683	1–>2268	106
cryICa7	*cryIC*	X96684	1–>2268	106
cryICb1	*cryIC(b)*	M97880	296–3823	48
cryIDa1	*cryID*	X54160	264–3758	42
cryIDb1	*prtB*	Z22511	241–3720	56
cryIEa1	*cryIE*	X53985	130–3642	115
cryIEa2	*cryIE*	X56144	1–3513	7
cryIEa3	*cryIE*	M73252	1–3513	82
cryIEa4		U94323	388–3900	47
cryIEb1	*cryIE(b)*	M73253	1–3522	81
cryIFa1	*cryIF*	M63897	478–3999	14
cryIFa2	*cryIF*	M73254	1–3525	80
cryIFb1	*prtD*	Z22512	483–4004	56
cryIGa1	*prtA*	Z22510	67–3564	56
cryIGa2	*cryIM*	Y09326	692–4210	96
cryIGb1	*cryH2*	U70725		12
cryIHa1	*prtC*	Z22513	530–4045	56
cryIHb1		U35780	728–4195	53
cryIIa1	*cryV*	X62821	355–2511	108
cryIIa2	*cryV*	M98544	1–2157	34
cryIIa3	*cryV*	L36338	279–2435	100
cryIIa4	*cryV*	L49391	61–2217	54
cryIIa5	*cryV159*	Y08920	524–2680	94
cryIIb1	*cryV465*	U07642	237–2393	100
cryIJa1	ET4	L32019	99–3519	25
cryIJb1	ET1	U31527	177–3686	116
cryIKa1		U28801	451–4098	52
cry2Aa1	*cryIIA*	M31738	156–2054	20
cry2Aa2	*cryIIA*	M23723	1840–3738	123
cry2Aa3		D86064	2007–3911	89
cry2Ab1	*cryIIB*	M23724	1–1899	123
cry2Ab2	*cryIIB*	X55416	874–2775	17
cry2Ac1	*cryIIC*	X57252	2125–3990	124
cry3Aa1	*cryIIIA*	M22472	25–1956	39
cry3Aa2	*cryIIIA*	J02978	241–2172	93
cry3Aa3	*cryIIIA*	Y00420	566–2497	41
cry3Aa4	*cryIIIA*	M30503	201–2132	65
cry3Aa5	*cryIIIA*	M37207	569–2500	22
cry3Aa6	*cryIIIA*	U10985	569–2500	1
cry3Ba1	*cryIIIB2*	X17123	25–>1977	101
cry3Ba2	*cryIIIB*	A07234	342–2297	85
cry3Bb1	*cryIIIBb*	M89794	202–2157	24
cry3Bb2	*cryIIIC(b)*	U31633	144–2099	23
cry3Ca1	*cryIIID*	X59797	232–2178	59
cry4Aa1	*cryIVA*	Y00423	1–3540	121
cry4Aa2	*cryIVA*	D00248	393–3935	95
cry4Ba1	*cryIVB*	X07423	157–3564	16
cry4Ba2	*cryIVB*	X07082	151–3558	112
cry4Ba3	*cryIVB*	M20242	526–3930	125
cry4Ba4	*cryIVB*	D00247	461–3865	95
cry5Aa1	*cryVA(a)*	L07025	1–>4155	102
cry5Ab1	*cryVA(b)*	L07026	1–>3867	67
cry5Ac1		I34543	1–>3660	76
cry5Ba1	PS86Q3	U19725	1–>3735	76
cry6Aa1	*cryVIA*	L07022	1–>1425	68
cry6Ba1	*cryVIB*	L07024	1–>1185	67
cry7Aa1	*cryIIIC*	M64478	184–3597	58
cry7Ab1	*cryIIIC(b)*	U04367	1–>3414	75
cry7Ab2	*cryIIIC(c)*	U04368	1–>3414	75
cry8Aa1	*cryIIIE*	U04364	1–>3471	29
cry8Ba1	*cryIIIG*	U04365	1–>3507	66
cry8Ca1	*cryIIIF*	U04366	1–3447	70
cry9Aa1	*cryIG*	X58120	5807–9274	104
cry9Aa2	*cryIG*	X58534	385–>3837	32
cry9Ba1	*cryX*	X75019	26–3488	97
cry9Ca1	*cryIH*	Z37527	2096–5569	57
cry9Da1	N141	D85560	47–3553	4
cry9Da2		AF042733	<1–>1937	122
cry10Aa1	*cryIVC*	M12662	941–2965	111
cry11Aa1	*cryIVD*	M31737	41–1969	21
cry11Aa2	*cryIVD*	M22860	<1–235	2
cry11Ba1	Jeg80	X86902	64–2238	19
cry11Bb1	94 kDa	AF017416		72
cry12Aa1	*cryVB*	L07027	1–>3771	67
cry13Aa1	*cryVC*	L07023	1–2409	90
cry14Aa1	*cryVD*	U13955	1–3558	77
cry15Aa1	34kDa	M76442	1036–2055	11
cry16Aa1	*cbm71*	X94146	158–1996	5
cry17Aa1	*cbm72*	X99478	12–1865	5
cry18Aa1	*cryBP1*	X99049	743–2860	126
cry19Aa1	Jeg65	Y07603	719–2662	86
cry19Ba1		D88381		87
cry20Aa1	86kDa	U82518	60–2318	61
cry21Aa1		I32932	1–3501	74
cry22Aa1		I34547	1–2169	76
cyt1Aa1	*cytA*	X03182	140–886	118
cyt1Aa2	*cytA*	X04338	509–1255	120
cyt1Aa3	*cytA*	Y00135	36–782	26
cyt1Aa4	*cytA*	M35968	67–813	30
cyt1Ab1	*cytM*	X98793	28–777	109
cyt1Ba1		U37196	1–795	78
cyt2Aa1	*cytB*	Z14147	270–1046	51
cyt2Ba1	*"cytB"*	U52043	287–655	35
cyt2Bb1		U82519	416–1204	15

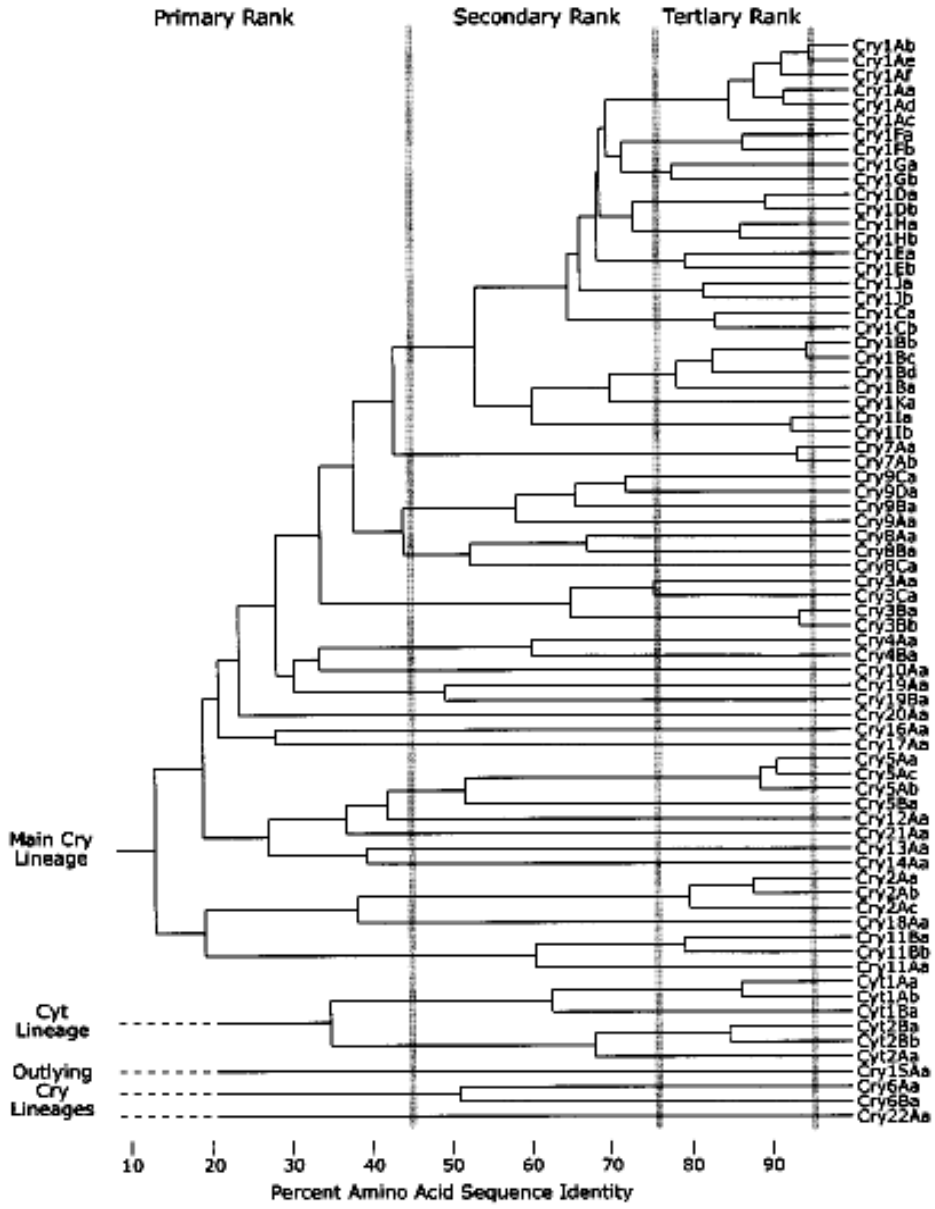


Figure 1. 2. Phylogram demonstrating amino acid sequence identity among Cry and Cyt proteins. This phylogenetic tree is modified from a TREEVIEW visualization of NEIGHBOR treatment of a CLUSTAL W multiple alignment and distance matrix of the full-length toxin sequences. The gray vertical bars demarcate the four levels of nomenclature ranks. Based on the low percentage of identical residues and the absence of any conserved sequence blocks inmultiple-sequence alignments, the lower four lineages are not treated as part of the main toxin family, and their nodes have been replaced with dashed horizontal lines (Crickmore *et al*., 1998).

1.2.2. Classes of Insecticidal Crystal Proteins

Cry proteins are specifically toxic to the insect orders Lepidoptera, Coleoptera, Hymenoptera, and Diptera Table (1.2.). Cyt toxins are mostly found in *Bt* strains active against Diptera. More than 3000 insect species within 16 orders were demonstrated to be susceptible to different insecticidal crystal proteins (ICPs). The search for new genes is an on-going effort worldwide. So far, the δ-endotoxins from the members of *Bt* comprise a group of 292 Cry and 22 Cyt proteins and are classified into Cry1 to Cry44 and Cyt1 to Cyt2 according to the degree of amino acid sequence homology (Stobdan *et al*., 2004; Huang *et al*., 2004)

Table 1. 2. Types of crystal proteins and their orders to which they are active.

Activity spectrum	**Crystal Protein**
Coleoptera	Cry3, Cry7, Cry8, Cry14, Cry 34, Cry 35, Cry 36, Cry 38
Diptera	Cry4, Cry10, Cry 11, Cry 16, Cry 19, Cry 20, Cry 24, Cry 25, Cry 27, Cry 29, Cry 30, Cry 39, Cry 40
Lepidoptera	Cry1, Cry9, Cry15
Lepidoptera and Diptera	Cry2
Hymenoptera	Cry22

The members of the three-domain family, the larger group of Cry proteins, are globular molecules containing three structural domains connected by single linkers. One particular feature of the members of this family is the presence of protoxins with two different lengths. One large group of protoxins is approximately twice as long as the majority of the toxins. The C-terminal extension found in the long protoxins is dispensable for toxicity and is believed to play a role in the formation of the crystal inclusion bodies within

the bacterium (de Maagd *et al*., 2001). Cyt toxins comprise two highly related gene families (Cyt1 and Cyt2) (Crickmore *et al*., 1998). Cyt toxins are also synthesized as protoxins and small portions of the N-terminus and C-terminus are removed to activate the toxin (Li *et al*., 1996).

1.2.2.1. Lepidoptera–Specific Cry Proteins

Lepidopteran-specific δ-endotoxins are produced by different *Bt* subspecies such as *Bt* subsp. *kurstaki*, *berliner*, *entomoicius* 6.01, *aizawai* 7.29, *thompsoni*, *sotto*, *fukuokaensis, canadensis*, and *galleriae*. The first characterized antilepidopteran δ -endotoxin was Cry1 produced by *Btk* HD1 (Dulmage, 1970). In 2004, Huang *et al*. isolated a novel *Bt* strain named as WB9 which is highly toxic to the lepidopteran *Plutella xylostella* and *Spodoptera exigua*, contains *cry*1A, *cry*1B, *cr*y1Cb, *cry*1Fa, *cry*1Ga genes and a *vip*3A gene. Besides Cry1 type of toxins, Cry2, Cry9 and Cry15 families are also toxic to lepidoptera. For examle, Cry15Aa1 and Cry9Da2 were isolated from *Bt thompsoni* and *Bt japonensis,* respectively (Brown and Whiteley, 1992; Wasano and Ohba, 1998). The encoded protoxins are 130-140 kDa molecular weight and are accumulated in the bipyramidal crystalline inclusions. However, Cry15 is found in crystals with a second apparently unrelated protein having a molecular weight of 40 kDa (De-Maagd *et al.*, 2003).

1.2.2.2. Diptera–Specific Cry Proteins

Antidipteran *Bt* strains commonly feature the presence of Cyt proteins with cytolytic and hemolytic activities (Guerchicoff *et al.,* 2001). The discovery of antidipteran *Bt israelensis* (*Bti*) in 1975 inagurated a new chapter in vector control (Goldberg and Margalit, 1977). The search for native strains with activity against dipteran species could have an impact on the control of

mosquitoes worldwide. Spherical shaped parasporal body of *Bti* comprises three types of Cry toxins; Cry4A (125 kDa), Cry4B (134 kDa) and Cry11A (67 kDa) and two types of Cyt toxins; Cyt1Aa (27 kDa) and Cyt2Ba (27-28 kDa) (Delecluse *et al*., 2000). Tests on single, solubilized and purified proteins showed that each of the crystal proteins are toxic to mosquitocidal larvae, but each one was not as toxic as the intact form alone (Becker and Margalit, 1993). Ibarra *et al*. (2003) characterized certain *Bt* isolates LBIT315, LBIT320, LBIT348, IB604 and 147-8906 from Latin America based on their insecticidal activities, SEM, SDS-PAGE and plasmid profiles as well as PCR analysis using novel general and specific primers for *cry* and *cyt* genes. They observed higher mosquitocidal activity than that of *Bti*. Cry11Ba1, Cry10Aa2, Cry20Aa1, Cry19Ba1, Cry25Aa1, Cry27Aa1, Cry29Aa1, Cry39Aa1 and Cry40Aa1, Cry30Ba1 and Cry24Ba1 were the proteins found from *Btk* HD1, *Bt jegathesan* 367, *Bti* ONR-60A, *Bt fukuokaensis, Bt higo, Bt jegathesan, Bt higo, Bt medellin, Bt aizawai, Bt sotto, Bt entomocidus,* respectively (GenBank Accession numbers: X86902, E00614, U82518, D88381, U88189, AB023293, AJ251977, BAB722016, BAD00052 and BAD32657)

1.2.2.3. Coleoptera–Specific Cry Proteins

Cry3A is highly toxic to *Leptinotarsa decemlineata*, one of the most important pest of coleoptera. This insect causes extensive damages on potato, tomatoes and eggplants. Other insect species belonging to Coleoptera order such as *Melolontha melolontha, Agelastica alni, Pyrrhalta luteola, Lasioderma serricorne* are also susceptible to anti-coleopteran *Bt* species. *Bt* BI256-82, the first identified strain as having activity against the larvae of Coleoptera order, was isolated from a dead pupa larvae of the yellow meal worm *Tenebrio molitor* by Huger in 1982 and the organism was described as *Bt* subsp. *tenebrionis* (*Btt*) by Krieg in 1983. Anti-coleopteran activity of *Btt* is related with the production of a flat, wafer shaped-rhomboidal crystal. To

date, several other anti-coleopteran *Bt* strains have been isolated and their toxicities have been evaluated. Hernstadt *et al.* (1986) identified *Bt san diego* which was later found to be identical to *Btt*. EG2158 was isolated and characterized by Donovan *et al.* (1988) to produce 73 kDa crystal protein toxic to coleoptera larvae. EG2838 (*Bt* subsp. *tolworthi*) and EG4961 (*Bt* subsp. *kumamotoensis*) were isolated and characterized by Rupar *et al*. (1991). The strains produced a 74 kDa polypeptide of CryIIIB (Cry3B) and Cry IIIB2 (Cry3Ba1), having a 94% sequence identity to each other (Donovan *et al*., 1992). 73 kDa protein of CryIIID (Cry7Aa) produced by *Btk* strain BT109P and active against larvae of Coleoptera (Lambert *et al*., 1992). Cry7Aa has an amino acid identity of 74 %, 61 % and 33 % with Cry3A, Cry3B and Cry3C, respectively. *Bt* LM63 and LM79 strains were isolated from soil samples by Chaufaux *et al.* (unpublished data) and identified as Coleoptera-active strains (Lecadet *et al*., 1992). Kaelin *et al.* (1994) analyzed 88 samples which were mostly consisting of dried leaf *Lasioderma serricorne* from different countries. 59% of the isolates produced rhomboidal crystals and gave 65 kDa protein bands which were similar to those residues, processed tobacco or finished product and dead tobacco beetles produced by Coleoptera-specific *Btt*.

Ellis *et al.* (2002) found new families of insecticidal crystal proteins with molecular masses of ca. 14 and 44 kDa produced by *Bt* isolates PS80JJ1, PS149B1, and PS167H2 that had cotton rootworm (genus *Diabrotica*) insecticidal activity. These binary Cry proteins designated as Cry34Aa1, Cry34Ab1 and Cry34Ac1 (the 14-kDa polypeptide components) and Cry35Aa1, Cry35Ab1 and Cry35Ac1 (the 44-kDa polypeptide components) were required for insecticidal activity.

A new *Bt galleriae* strain containing novel *cry*8 gene was discovered and shown to be highly toxic against scarab beetles such as *Anomola cuprea*

(Asano *et al.,* 2003). Anti-Coleopteran Cry7Ab2 and Cry14Aa1 proteins were also isolated from *Bt kumamotoensis* 867 and *Bt sotto* PS80JJ1, respectively (GenBank Accession numbers: U04368 and U13955). In our laboratory, a cry3 producing local isolate of *Bt* Mm2 was identified. *Bt* Mm2, with its 65 kDa toxin, had insecticidal activity against the larvae of *Melolontha melolontha*, *Agelastica alni*, *Leptinotarsa decemlineata* and *Amphimallon solstitiale*. The sequence of the cry3Aa gene was determined (GenBank Accession no. AY882576 Mm2 was named as cry3Aa11 by the B. thuringiensis Pesticidal Crystal Protein Nomenclature Committee and became a new member of B. thuringiensis toxins. (Kurt *et al*., 2005a).

1.2.2.4. Hymenoptera–Specific Cry Proteins

Cry22 proteins were initially identified in *Bt* strains as active on ants (Order: Hymenoptera). The proteins have molecular weights of 75 to 86 kDa and Cry5 toxins were also reported to be active against the ant *Monomorium pharaonis* that belongs to the order Hymenoptera (Payne *et al*., 1997). Cry5A toxin from *Bt* strain PS86Q3 was active against at least one hymenopteran species, *Diprion pini.* Strain PS86Q3 contained a long bipyramidal crystal composed of five proteins of 155, 135, 116, 97 and 58 kDa., respectively. The 155 kDa protein was proven to be Cry5B toxin by N-terminal sequencing, whereas the other proteins were Cry5A toxins. Thus, it was possible that 135, 116, 97 and 58 kDa proteins represent proteolytic fragments of the same protein, since PCR analysis demonstrated that this strain harbors only *cry5*Ac and *cry5*Ba genes. The treatment with proteases present in *D. pini* or *Cephalcia abietis* midgut content produced a single 75 kDa protein (Garcia-Robles *et al*., 2001).

1.2.3. Structural and Sequential Similarities Among Cry Toxins

When the structural and sequential similarities are considered, conserved amino acid sequences drew attention among most Cry toxins (Hofte and Whiteley, 1989). Alignment of the Cry toxins reveals the presence of five conserved sequence blocks common to a large majority of proteins as shown in Figure 1.3. The amino acid sequence which starts by the end of the conserved block 5 and continues down to C-terminal region is the cleavage part by the proteases during crystal protein activation. The function of block 5 is largely structural, rather than related to activity. Blocks 1-4 are involved in ion channel function or toxin stability.

Within different classes of toxins the number of common blocks can also differ. The group consisting of Cry1, Cry3, Cry4, Cry7 to Cry10, Cry16, Cry17, Cry19, and Cry20 contains all five of the core blocks. A second group consisting of Cry5, Cry12 to Cry14, and Cry21 contains blocks 1, 2, 4, and 5. Hovewer, Cyt1, Cyt2, Cry6, Cry15, and Cry22 have no recognizable homologs to the conserved blocks (Schnepf, 1995; Schnepf *et al*.,1998; De-Maagd *et al.*, 2001).

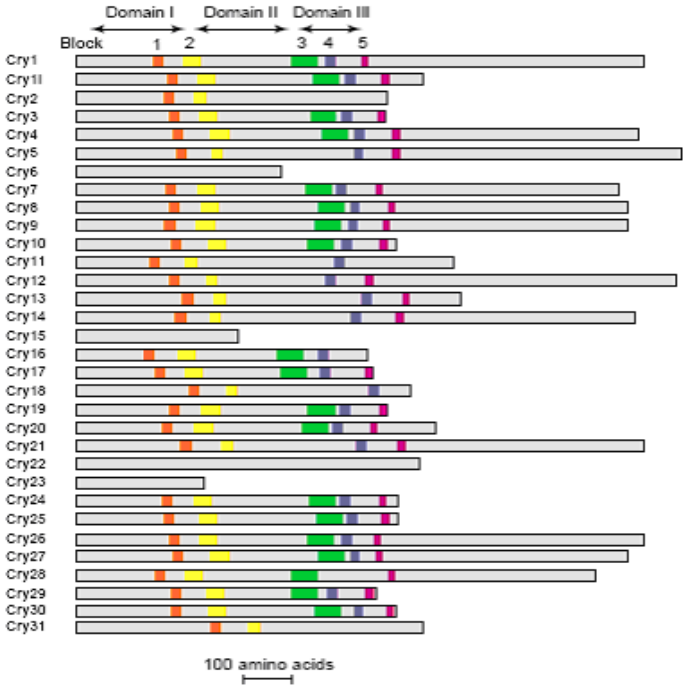


Figure 1. 3. Five conserved sequences of insecticidal crystal proteins.

Cry3A and Cry1Aa, in contrast to Cyt2A, both possess three domains, as shown in Figure 1.3. Domain I consists of a cluster of seven antiparallel δ -helices in which helix 5 is encircled by the remaining ones (Schnepf *et al.,* 1998). Domain II consists of three antiparallel β-sheets joined in a typical "Greek key" topology, arranged in a so-called β -prism fold (Sankaranarayanan *et al*., 1996; Shimizu and Morikawa, 1996). Domain III consists of two twisted, antiparallel β-sheets forming a α-sandwich with a "jelly roll" topology (Li *et al*., 1991).

To date, the tertiary structures of six different three-domain Cry proteins, Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb, Cry4Aa and Cry4Ba have been determined by X-ray crystallography (Bravo *et al*., 2007) (Figure 1.4). All these structures display a high degree of similarity with a three-domain organization, suggesting a similar mode of action of the Cry three domain protein family. The N-terminal domain (domain I) is a bundle of seven a-helices in which the central helix-a5 is hydrophobic and is encircled by six

other amphipathic helices; and this helical domain is responsible for membrane insertion and pore-formation. Domain II consists of three antiparallel b-sheets with exposed loop regions, and domain III is a b-sandwich.

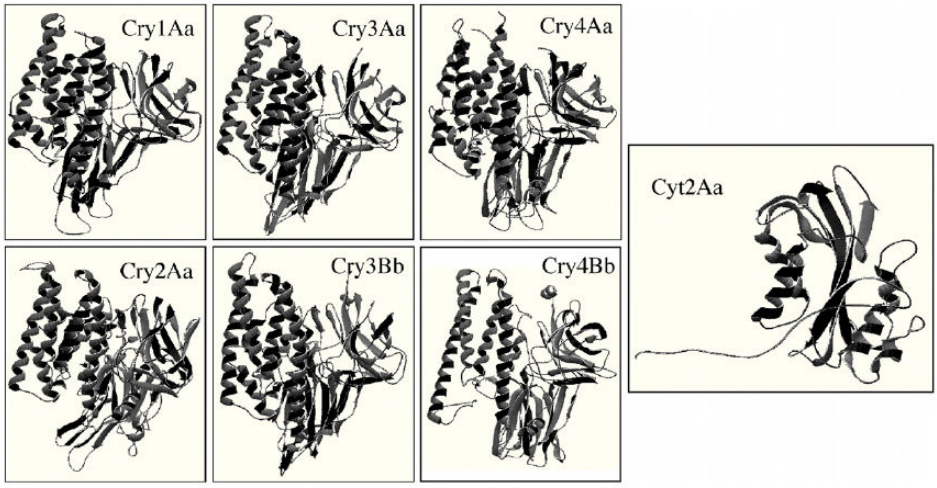


Figure 1. 4. Three dimensional structures of insecticidal toxins produced by *Bacillus thuringiensis* Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb, Cry4Aa, Cry4Bb and Cyt2A. The high specificity of δ-endotoxins could limit the widespread utilization of *Bt* bioinsecticides to control large varieties of agricultural pests (Zourai *et al.*, 2002).

1.3. Vegetative Insecticidal Protein (Vip) Toxins

In recent years, Vip toxins, have been described which are expressed in vegetative cells of *Bt* and then secreted from the cell (Estruch *et al*., 1996). In contrast to the crystal toxins which can be effective in the absence of a viable *Bt* cell, the Vip toxins clearly require a vegetative growing cell to exert their pathogenic effect.

1.4. Mode of Action of *Bt* Toxins

While there is considerable genetic sequence variation between the classes of Cry toxins, there is a high level of conservation within a set of five functional blocks, and comparisons of the three-dimensional structures from Cry1, Cry2, and Cry3 reveal that they share a high degree of structural similarity (de Maagd *et al*., 2001). Because of the similarity in conserved domains and structure, it is also likely that they also share a high degree of functional similarity. The differences in sequence undoubtedly are responsible for insect order and binding site specificity. The general model for the function of Cry proteins is based on Cry1 because more work has been done on it than any other member of this class of protein (Aronson and Shai, 2001; Gringorten, 2001).

Phytophagous insects require an extremely alkaline midgut; the pH in Lepidopteran larvae is commonly found to be in the range of 10 to 11. The high pH prevents tannins from complexing with and inactivating digestive enzymes. By dissociating tannins from leaf proteins, the digestibility of the leaf tissue is enhanced. Goblet cells in the midgut epithelium play a critical role in the maintenance of pH by secreting potassium carbonate into the lumen of the midgut. These cells are also central to maintaining high potassium concentrations with an energy-dependent potassium pump that pushes K^+ from the hemolymph and columnar epithelial cells back into the gut lumen. The two gradients, high pH and K^+ concentration, in the lumen are used by amino acid symporters for the absorption of nutrients into columnar cells of the midgut epithelium (Gringorten, 2001).

For *Bt* to successfully attack and colonize a larva, it must defeat each of the insect's defenses as it progresses through the steps of its pathology. After ingestion of the *Bt* crystal inclusion, toxicity is dependent on a complex

process that requires multiple steps. These include solubilization of the crystal proteins, proteolytic processing of the protoxin to the active form, high affinity binding with the midgut receptor, and the irreversible insertion of the toxin into the membrane (Jenkins *et al*., 2000). The progression of *Bt* toxicity is summarized in Figure 1.5.

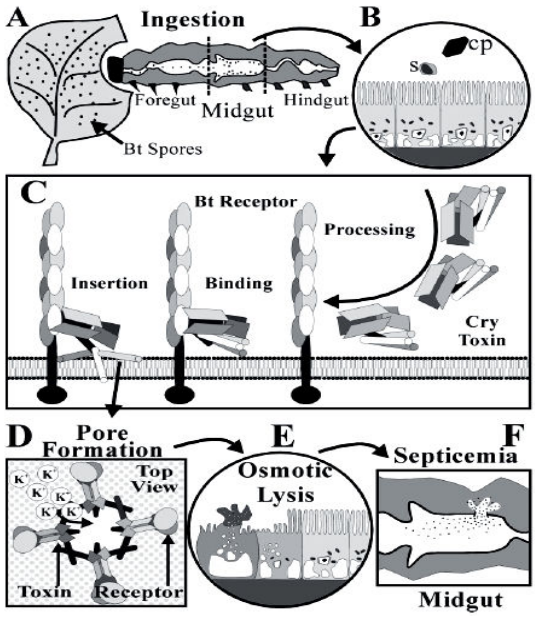


Figure 1. 5. Mechanism of Cry protein toxicity (Whalon and Wingerd, 2003). A: Ingestion of spores or recombinant protein by phytophagous larva. B: In the midgut, endotoxins are solubilized from *Bt* spores (s) and inclusions of crystallized protein (cp). C: Cry toxins are proteolytically processed to active toxins in the midgut. Active toxin binds receptors on the surface of columnar epithelial cells. Bound toxin inserts into the cellular membrane. D: Cry toxins aggregate to form pores in the membrane. E: Pore formation leads to osmotic lysis. F: Heavy damage to midgut membranes leads to starvation or septicemia.

There is a suite of specific characteristics that allow *Bt* to be a particularly effective pathogen. In order to pass through the foregut, *Bt* must be present as a very small spore rather than in its larger vegetative state, which would be more susceptible to damage and exclusion from the midgut. In the midgut, the pH is far too alkaline for the spore to germinate, but the insecticidal

crystal proteins circumvent this aspect of the insect defense. *Bt* species contain a variety of endotoxins and helperfactors (Kumar and Venkateswerlu, 1998; Estruch *et al*., 1996; Agaisse *et al*., 1996), of these, the δ-endotoxins play the most critical role in *Bt* mediated toxicity. The Cry proteins solubilized from crystal inclusions of the *Bt* spore are inactive in their pro-toxin form (Choma *et al*., 1991). Before toxicity can occur, the pro-toxin must be proteolytically processed. This requires the high pH found in the midgut as well as digestive enzymes from the insect. Activation involves the removal of both the carboxyl terminal and the amino terminal ends of the protein (Gringorten, 2001). Once activated, the Cry toxin diffuses from the lumen through the periplasmic membrane into the endoperiplasmic space. The fully processed and active Cry toxin now has access to the surface of the columnar epithelial cells (Hill and Pinnock, 1998).

At the cell surface, a critical handshake occurs as the Cry protein binds to its receptor. Aminopeptidases, which are involved in digestion and cell adhesion molecules similar to cadherins function as receptors for the cry proteins (Vadlamudi *et al*., 1995; Luo *et al*., 1997; Jenkins and Dean, 2001). Binding of the Cry proteins is thought to occur at a membrane proximal region of these membrane bound proteins. Evidence from receptor binding studies has demonstrated that some Cry proteins bind to more than one site on the cell surface (Jurat-Fuentes *et al*., 2002). In addition, glycosylation is critical for some of the interactions. As a result, resistance to one Cry toxin does not guarantee universal resistance to all Cry proteins, although the presence of multiple resistance alleles in one individual has been shown to contribute to cross-resistance to multiple Cry toxins (Sayyed *et al*., 2000; Herrero *et al*., 2001; Jurat-Fuentes *et al*., 2002).

As mentioned earlier the Cry protein is composed of three distinct domains. After binding, domain I goes through a rearrangement similar to that of

opening an umbrella. The three pairs of α-helices in domain I open and insert into the membrane, placing domain III at the membrane surface over the inserted helices. Insertion of the cry protein appears to be irreversible (Li *et al*., 2001). Next, aggregation of inserted Cry proteins occurs, resulting in the formation of pores. The pores are most likely tetramers and form a K^+ selective ion channel (Gringorten, 2001).

The formation of this channel immediately leads to two very significant and detrimental physiological changes in the insect. First, the K^+ gradient in the epithelial cells is disrupted, which leads to an increase in hemolymph K^+ concentrations. Second, the pH gradient is disrupted leading to a decrease in the pH of the midgut lumen and an increase in the hemolymph pH. Ultimately, the affected cells are destroyed by the high pH of the midgut and osmotic lysis. As a result of the lysis of cells in the midgut epithelium, the spore is allowed to germinate in a nearly neutral environment, bathed in the nutrients from ruptured cells. Most insects are not killed directly by the effects of the toxin but die as a result of rapidly induced gut paralysis and feeding inhibition, and subsequent starvation or septicemia (Gringorten, 2001; Whalon and Wingerd 2003).

1.5. Applications of Cry toxins

Three major applications of *Bt* toxins have been achieved: (i) in the control of defoliator pests in forestry, (ii) in the control of mosquitoes that are vectors of human diseases, and (iii) in the development of transgenic insect-resistant plants (Bravo et al., 2007).

One of the most successful applications of *Bt* has been the control of lepidopteran defoliators, which are pests of coniferous forests mainly in Canada and United States. In both countries, the control of forests defoliators

relies mostly on the use of *Bt* strain, HD-1, producing Cry1Aa, Cy1Ab, Cry1Ac and Cry2Aa toxins. Successful application of *Bt* is highly dependent on proper timing, weather conditions and high dosage of spray applications. These factors combine to determine the probability of larvae ingesting a lethal dose (van Frankenhuyzen, 2000; Bauce *et al*., 2004). The use of *Bt* in the control of defoliators has resulted in a significant reduction in the use of chemical insecticides for pest control in the forests (Bravo *et al*., 2007).

As mentioned previously, *Bti* is highly active against disease vector mosquitoes like *Ae. aegypti* (vector of dengue fever), *Simulium damnosum* (vector of onchocerciasis) and certain *Anopheles* species (vectors of malaria). Its high insecticidal activity, the lack of resistance to *Bti*, the lack of toxicity to non-target organisms and the appearance of insect-resistant populations to chemical insecticides resulted in a rapid implementation of *Bti* as an alternative control method of mosquito and black fly populations (Becker, 2000). In 1983, a control program for the eradication of onchocerciasis was launched in eleven countries of Western Africa using *Bti* since *S. damnosum* populations had developed resistance to larvicidal organophosphates. Presently, more than 80% of this region is protected by *Bti* applications and 20% with the chemical larvicide, temephos. Furthermore, control of onchocerciasis has protected over 15 million children without the appearance of black fly resistance to *Bti* (Guillet *et al*., 1990). This success of vector control using *Bti* will certainly increase its use around the world. However, the low activity of *Bti* to certain vector mosquitoes, mainly Anophelines, will require the isolation of other *Bt* strains with novel cry genes more effective against these important disease vectors.

The development of transgenic crops that produce *Bt* Cry proteins has been a major breakthrough in the substitution of chemical insecticides by environmental friendly alternatives. In transgenic plants the Cry toxin is

produced continuously, protecting the toxin from degradation and making it reachable to chewing and boring insects. Cry protein production in plants has been improved by engineering *cry* genes with a plant biased codon usage, by removal of putative splicing signal sequences and deletion of the carboxy-terminal region of the protoxin (Schuler *et al*., 1998). The use of insect resistant crops has diminished considerably the use of chemical pesticides in areas where these transgenic crops are planted. Interestingly, the use of *Bt*-cotton in countries like China, Mexico and India showed that the use of this *Bt*-crop had a significant positive effect on the final yield and a reduction in the use of chemical pesticides, since in these countries the yield loss is mainly due to technical and economical constrains which are overcome in part by the use of insect-resistant crops (Qaim and Zilberman, 2003; Toenniessen *et al*., 2003; Bravo *et al*., 2007).

1.6. Strategies to Improve *Bt* and Toxin Yields

1.6.1. Optimization of Nutritional and Cultural Conditions

Bt is the most inexpensive of all microbial insecticides in terms of production costs. Still, high protein yields for optimization of *Bt* fermentations are required to make its production cost-competitive with synthetic insecticides. Systematic studies on the physiological and nutritional requirements of *Bt* especially those required for maximum yield of ICPs are still limited (Sachidanandham *et al*., 1997). Fermentation and media optimization for each strain put into commercial production is needed as different strains may have different responses to nutritional and cultural conditions (Kaur, 2000). The culture conditions of *Bt* are optimized to achieve not only high cell densities, but also high crystal protein concentration and high toxicity (Dulmage *et al*., 1990). Previously, it was believed to be important to end up with high spore counts however, Rossa and Mignone (1993) reported that a

good spore count did not lead to high larvacidal potency in *Bti.* It was later shown that bacterial spore counts do not necessarily reflect the insecticidal activity of a *Bt* strain or *Bt* product because the number and amount of crystal protein per bacterial cell can vary (Paramatha, 2000). In some countries like Japan, products of *Bt* biopesticides with viable *Bt* spores are not authorized. Dissemination and persistence of *Bt* spores in locations where sericulture is an economic activity is a matter of concern due to toxicity to economically important insect, such as the toxicity of *cry*1Aa gene carrying spores to silk worms. Thus, from a commercial perspective, *Bt* strains which produce crystals but not spores, and thereby nonpersisting in nature are desirable (Kaur, 2000).

For *Bt* fermentations carbon source is very important. *Bt* uses sugars, usually glucose, fructose, maltose, ribose, molasses, starch, dextrin, wheat flour and inulin, producing acid during the fermentation (Nickerson and Bulla 1974; El Bendary, 2006). The presence of a carbon source in growth medium can repress expression of certain genes and operons indirectly related with toxin formation (Bruckner and Titgemeyer, 2002). The excessive glucose concentration may prolong the vegetative growth and also minimize the toxin yield. For example, Bhatnagar (1998) showed that Cry4A toxin of *Bti* was not synthesized when the medium contained 0.4 % glucose. In the study of İçgen *et al.* (2002b), it was found that the carbon sources such as sucrose, lactose and inulin supported the production of Lepidoptera-specific Cry1 and Cry2 toxins by *Bt* 81. The replacement of glucose with sucrose greatly increased the yields of Cry4Ba and Cry11A toxins (Özkan *et al*., 2003).

With respect to the nitrogen sources suitable for *Bt* production, the overwhelming majority of literatures revealed the inability of most of *Bt* varieties to utilize inorganic nitrogen source as a sole nitrogen source in the growth medium. Instead, at least one amino acid particularly glutamate,

aspartate, valine, leucine, serine or threonine has to be added in order to allow growth of the organism in a minimal medium. However, cysteine and cystine amino acids showed clear inhibitory effect on growth, sporulation and toxin formation by *Bt* (El-Bendary, 2006). It was suggested that the depletion of nitrogen sources triggers sporulation and toxin expression. Inorganic nitrogen compounds, such as ammonium sulfate are not usually sufficient to support the growth of *Btk* and organic nitrogen sources such as meat peptone, fish meal and soybean flour are required for rapid growth (Yang and Wang, 2000). Among different organic and inorganic nitrogen compounds tested, peptone was found to be best source supporting the optimum Cry1 and Cry2 δ -endotoxin formation (İçgen *et al*., 2002b).

Another important component for the production of crystal protein is potassium ion. Wakisaka *et al* (1982) detected an increased crystal protein production in the presence of potassium salts. However, when potassium salts were replaced with the same salts of sodium, the same effect was not seen. Besides this, Bhatnagar (1999) reported a corresponding increase in the Cry4A specific mRNA in the presence of inorganic phosphate which stimulated 135 kDa protoxin synthesis by *Bti* cells. Phosphate is considered to be essential for not only protein expression, but also for cell growth as the concentration of phosphate varies during the toxin-formation phase (Yang and Wang, 2000). Previous studies conducted in our laboratory with *Bt* 81 (İçgen *et al*., 2002a) and *Bt* HD500 (Özkan *et al*., 2003) high yields of anti-lepidopteran and anti-dipteran δ-endotoxins were obtained with 50-100 mM inorganic phosphate among the concentrations tested. Moreover, it is showed that inorganic phosphate had the most striking effect on Cry3Aa δ-endotoxin production. Iron effect was unique to *Bt* strain Mm2 producing cry3Aa11 whereas Pi effect was common to the biosynthesis of Cry3Aa-type toxins. Stimulation of toxin synthesis by Pi did not represent a relief from glucose repression (Kurt *et al*., 2005b). Bacterial toxins possess many attributes of secondary metabolites and their yield is known to be greatly

affected by trace metals (Rose, 1979). Mn is the most important key metal co-regulating secondary metabolism and differentiation in bacilli (Weinberg, 1977). Sikdar *et al.* (1991) reported on the mineral requirements of *Bti* for production of the mosquidocidal δ -endotoxin and found that Fe, Mn and Cu were required for the production of crystal protein while Mo had an inhibitory effect. They also obtained no direct relationship between cell growth and toxin production by comparing optimum levels of metals for both. In the study of İçgen *et al.* (2002 a), the toxin yield was almost zero when Mg was omitted from the medium, indicating that Mg was the most critical trace element for antilepidopteran Cry protein synthesis by *Bt* 81. The stimulatory effect of Cu was seen between the range of 10^{-6} to 10^{-7} M. Ca and Zn had no effect on toxin production. However, Ca favored and Zn, Fe and Cu negatively influenced the synthesis of antidipteran toxin production by *Bti* HD500 (Özkan *et al*., 2003). Sachidanandham *et al.* (1997) identified the amino acid requirements for a stable and enhanced production of crystal proteins by *Bt* subsp. g*alleriae* (*Btg*). In the study, several amino acids resulted in a better carbon utilization as well as improving the stability and volumetric productivity of *Btg* biomass.

The growth of *Bt* occurs in the pH range of 5.5–8.5 (Rowe and Margaritis 1987; Içgen *et al*. 2002b and Özkan *et al*. 2003). The usual initial pH is 6.8–7.2; decreasing to 5.8 as acetate is released, then rising to 7.5–8 as it is consumed (El Bendary, 2006). The normal temperature for growth and toxin production of *Bt* is 30 °C. Özkan *et al*. (2003) found that Cry4Ba synthesis by *Bt* HD500 was the best when the organism was grown at 25 °C, whereas Cry11Aa synthesis was optimal at 30 °C.

1.6.2. Molecular Approaches

Several techniques, such as genetic recombination and construction of engineered *Bt* strains have been used to improve the insecticidal properties of *Bt* strains (Sanchis *et al.*, 1996; Kaur, 2000).

Expression of *cry* genes can be enhanced by altering the elements regulating transcription and translation. Cry3Aa protein production was enhanced 2.5 fold in an asporogenous *Bt* strain, suggesting sporulation-independent transcription of *cry*3Aa (Malvar *et al*., 1994). The expression of cry3Aa was increased in sporulation-negative *spo*A *B. subtilis* mutants (Agaisse and Lerecluse, 1994 a). Moreover, Sanchis *et al.* (1996) reported the accumulation of Cry1C toxin in large amounts by a *spo*0A mutant expressing the *cry*1C coding sequence from the sporulation-independent *cry*3A promoter.

20 kDa helper proteins encoded by the *cry*2A and *cry*11A operons enhanced synthesis and stability of *cry*2A by acting as molecular chaperons (Ge *et al*., 1998). Transcipt stability is another important factor for Cry synthesis. As substantial increase in 65 kDa Cry1 was obtained using the STAB-SD sequence of the *cry*3A gene (Agassie and Lereclus, 1996). The use of dual *cyt*1Aa promoter along with STAB-SD sequence resulted in several-fold increase in the expression of *cry*3Aa gene. Besides, it was shown that *cyt*1 promoters in combination with the *cry*3A STAB-SD enhanced Cry2A and Cry11A yields almost 5 and 1.3 folds, respectively (Park *et al*., 1998).

An increase in irreversible binding to receptors was correlated with increase in toxicity as well. Amino acid residues involved in receptor recognition, membrane insertion and toxicity can be substituted through in vitro mutagenesis (Smedley and Ellar, 1996). A combination of mutations in the a

8 loop and loop 2 in domain II of Cry1Ab resulted in a 32-fold increase in toxicity to gypsy moth (*Lymantaria dispar*) over the wild type protein (Rajamohan *et al*., 1996). Wu *et al.* (2000) used site-directed mutagenesis to modify *Bt cry*3A gene in amino acid residues 350-354 and showed a significantly improved toxicity against *Tenebrio molitor*.

Insecticidal activity of *Bt* strains can be further improved by elimination of activities that reduce Cry production. During sporulation, *Bt* synthesizes proteolytic enzymes for processing of non-toxic protoxins to smaller insect-active toxins. However, they may also process and degrade crystal protein resulting in yield reduction (Kaur, 2000; Tan and Donovan, 2000). Donovan *et al.* (1997) by using a gene deletion strategy, showed that neutral protease A, a sporulation-specific protease, contributed to degradation of crystal proteins.

1.6.3. Renewable sources

There is a growing interest in the search for alternate high yielding, ubiquitous and cost effective raw materials to produce fermentation products. Several studies were conducted to decrease the cost of fermentation by employing low-cost media components. For example, Poopathi *et al*. (2002) suggested efficient use of a potato-based culture medium for the industrial production of *Bt* israelensis**.** Vora and Shetna (1999) reported enhanced growth, sporulation and toxin yields by *Bt* kurstaki when defatted soybean and ground nut seed meal extracts were used with the supplementation of cystine.

The human industrial activities, inevitably, generate industrial wastes, consisting of inorganic and organic materials, discharged from factories, fisheries, poultries and food processing industries. Gruel and fish meal

media were also investigated for the growth of both diptera-active and lepidoptera-active *Bt* strains. Their use to cultivate anti-lepidopteran subspecies was found to be more beneficial than their use for the anti-dipteran supspecies which displayed decreased toxicity (Zouari *et al*., 2002). In recent years, waste water sludge as a raw material for the production of *Bt* kurstaki was also evaluated (Lachhab *et al*., 2001; Montiel *et al*., 2001; Vidyarthi *et al*., 2002) and thought to minimize *Bt* production costs and reduce the quantity of sludge for disposal. Sludge contains nutrients required for growth of microorganisms. Therefore, use of sewage sludge as a raw material for the production of *Bt*-based bioinsecticide is an alternate novel method of sludge utilisation/disposal (Yezza *et al*., 2006). The use of sludge for *Bt* production is also advantageous in the reduction of usage of chemical pesticides.

Since broiler litter contains approximately 30% carbohydrate (mostly crude fiber in pine wood shaving broiler litter) and 30% crude protein on a dry basis, this material is a potential bioresource (Adams *et al*., 2002). Moreover, broiler litter is widely available, with an estimated 5.6 million metric tones of broiler litter generated in the US annually (Snipes, 1997). There is no statistical data available in Turkey related with the amount of available broiler litter. Only the number of poultry animals is known, about 318 million , as reported by Turkish Statistical Institute in 2005. Since it is a biologically produced material and contains crude protein and other nutrients, poultry litter has requisite characteristics of a good substrate for microbial growth. Solid state fermentation using broiler litter to produce *Bt*-based bioinsecticide was the first report (Capalbo and Morales, 1997) followed by the study of Adams *et al*. (1999) who described the use of broiler litter in batch submerged fermentation. While antimicrobial compounds exist within litter that can inhibit growth of some microorganisms, careful selection of microbial species and/or litter physical characteristics could ameliorate inhibition problems. Moreover, it was postulated that the use of litter as a substrate to produce biocontrol

agents and as a delivery system for agents requiring soil inoculation has considerable promise.

1.7. Present Study

Although the structure of the *Bt* toxins, their action mechanisms, molecular biology and genetics of toxin biosynthesis are well documented, there are relatively few literature reports about its fermentation and industrial production. The importance of the medium components and other variables required during fermentation process is very well known (Farrera et al., 1998).

The aim of the present study was to compare toxin biosynthesis by three different *Bt* subspecies, namely *Bt kurstaki* (strain 81), *Bt israelensis* (strain HD500) and *Bt tenebrionis* (strain 3203), producing lepidoptera- and diptera-specific Cry1 and Cry2, diptera-specific Cry4Ba and Cry11Aa and coleoptera-specific Cry3Aa toxins, respectively, as a function of nutrient composition of cultivation medium. The effects of varying glucose and inorganic phosphate concentrations were determined first, and then, a low cost medium for high toxin production was tried to be devised by using certain agroindustrial and renewable raw materials and wastes.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial Strains

The bacterial strains and plasmids used in this study and their sources are listed in Table 2.1. The strains were streaked onto LB agar (Appendix A), subcultured monthly and stored at + 4 ^{o}C. For long term maintenance of *Bacillus* strains, stock solutions were stored at - 80 ^{o}C in Luria broth covered with the addition 20 % glycerol.

Seed culture of *Bt* strains were prepared according to the procedure adopted from Stahly *et al* (1992). The *Bt* strains were grown to mid-log phase (ABS_{600}= 0.5). A 100 μL aliquot from each culture was transferred into a 100 mL DSM medium and left to grow until OD_{600} became 0.5. Then 100 μL of the culture was diluted 50 folds by adding 4.9 mL of 20 % glycerol (v/v). The diluted culture was divided in Eppendorf tubes as 200 μL aliquots and stored at – 80 °C.

Table 2. 1. A list of bacterial strains used in the present study.

No.	Strain	*Bt* serovar	Source and reference
1	81	*kurstaki*	Local isolate
2	3203	*morrisoni*	Prof. Feruccio Gadani, Philip Morris Europe S.A., Neuchatel, Switzerland; Kaelin *et al.* (1994)
3	HD500	*israelensis*	BGSC

2.2. Culture Media

The composition and the preparation of the culture media are given in Appendix A.

2.3. Buffers and Solutions

Buffers, solutions, their composition and preparation are listed in Appendix B.

2.4. Chemicals and Enzymes

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

2.5. Fermentation and Construction of Growth Curves

For inoculum preparation, the glycerol stock of each *Bt* strain was first inoculated (0.1%) into 50 mL of DSM in a 250 mL flask and cultured overnight by shaking at 30^{o}C at 200 rpm. 0,5 mL aliquots of such overnight cultures were used to inoculate 50 mL of fresh DSM D in 250 mL Erlenmayer flasks to start fermentation by shaking at 30^{o}C at 200 rpm for 24 to 72 h. Considering the inoculation time as the time zero, samples were taken from the culture at two hour intervals and used for quantitative determination of growth which was measured spectrophotometrically as absorbance at 600 nm. Viable counts were also made and cell concentrations were determined as 'Colony Forming Units/mL' (CFU/mL).

2.6. Determination of Sporulation Frequency

The samples were taken from the bacterial cultures grown in DSM by shaking at 30 °C. 1 mL aliquots of the cultures were serially diluted in tubes containing physiological saline solution. Colony forming units were determined after overnight incubation of inoculated Nutrient agar plates with prescribed volume of the diluted tubes at 30 °C. For spore count, the tubes were heated at 75 °C for 15 min in a waterbath, were cooled, dilutions were placed onto Nutrient agar plates and incubated at 30 °C for 16 h. Sporulation frequency was expressed in terms of the ratio of the numbers of heat-resistant spores per mL to the ratio of the number of viable cells per mL (Özcengiz and Alaeddinoğlu, 1991).

2.7. Protein Extraction

A slightly modified procedure of Armelle (1991) was used for protein extraction. 5-10 mL of 72 h cultures were centrifuged at 4 000 rpm (1500g) for 20 min. The pellet was resuspended in 500 μL of 1M NaCl. This mixture was transferred into an Eppendorf tube and centrifuged at 7 000 rpm for 7 min. The pellet was resuspended in 250 μL of TE buffer (Appendix B) and centrifuged at 7 000 rpm for 7 min. The last step was repeated after which the pellet was resuspended in a 250 μL dH_2O to obtain a homogenous suspension. 150 μL of 10 $mg.mL^{-1}$ 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 and then the tube was vortexed for 30 sec. It was centrifuged at 6 000 rpm for 10 min and 100 μL of 0.2 % SDS solution was added to the pellet. For denaturation, 40 μL of the protein sample was mixed in an Eppendorf tube with 10 μL gel loading buffer (Appendix B). This sample was incubated at

90 °C for 7 min. for three times and placed onto ice until it cools. The final native and denatured samples were stored at – 20 °C.

2.8. Determination of Protein Concentration

Protein concentrations were measured by the Bradford quantification method (1976). The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 at 595 nm when binding to protein occurs. Assay reagent was made by dissolving 100 mg of Coomassie Blue G250 in 50 mL of 95 % ethanol. The solution was then mixed with 100 mL of 85% phosphoric acid and made up to 1 L with distilled water. The reagent was filtered through Watman No. 1 filter paper. Bovine serum albumin (BSA) was used as the standard for preparation of protein calibration curve. Volumes of 10, 15, 20, 30 and 50 μL of 1 $mg.mL^{-1}$ standard protein, BSA were added to tubes and volumes were adjusted to 500 μL with water. 500 μL of distilled water was added into a tube as reagent blank. 4.5 mL of assay reagent was added to each tube and mixed gently, but thoroughly. A standard curve of absorbance versus micrograms protein was prepared and the amounts of proteins were determined from the curve. The 20μg of protein was loaded on the SDS-PAGE gels.

2.9. SDS-Polyacrylamide Gel Electrophoresis

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

The SDS-polyacrylamide gels were prepared as described below (Laemmli, 1970):

Table 2. 2. Ingredients of SDS-PAGE

	Stacking Gel 0.125 M Tris, pH 6.8	**Separating Gel** 0.375 M Tris, pH 8.8
Monomer concentration	**4.5%**	**12%**
Acrylamide/bis dH_2O 1.5 M Tris-HCl, pH 8.8 0.5 M Tris-HCl, pH 6.8 10% (w/v) SDS 10% Ammonium persulphate (fresh) TEMED	1.3 mL 6.1 mL - 2.5 mL 100 μL 50 μL 10 μL	4mL 3.35 mL 2.5mL - 100 μL 50 μL 5 μL
TOTAL MONOMER	10 Ml	10 mL

2.10. Staining of the SDS-Polyacrylamide Gel

Proteins were visualized by Coomassie Blue R-250 staining of the gels. After electrophoresis, the gel was soaked in 200 mL of freshly prepared Coomassie blue stain (Appendix B) for 1 h at room temperature. The gel was then destained by keeping it in destaining solution (Appendix B) for at least 24 h.

2.11. Protein Quantification

Gels were photographed by Vilber Lourmat Gel Imaging System and amount of the proteins were detected by Bio-Profile Image Analysis Software (Vilber Lourmat).

2.12. Preparation of Low Cost Media

2.12.1. Preparation of Media Based on Wastewater Sludge

The wastewater sludge used in this study was obtained from METU Membrane Bioreactor Center wastewater treatment plant. The sludge was a mixture of primary and secondary sludges. The samples were obtained in glass containers. The sludge preparation was adopted from the protocol described by Brar et al., 2005.

As nutrient content of manure of sludge decreases by time drastically, the samples had to be processed immediately The sludge was dried at 80^{o} C till no wet particles remained in order to stop microbial activity as the microbial activity causes loss of nutritional value. The dried bulk sludge was ground to a fine homogenous powder. The powders were stored at 4^{o} C until use. 10 to 25 g of sludge powder was suspended in 1 L of dH_2O (the resulting suspension was filtered through a Whatman No.1 paper whenever indicated in the text). The pH of the suspension was adjusted to 7.2 and the resulting sludge-based medium was next sterilized by autoclaving at 121^{o} C for 15 min to prevent sludge deterioration during storage.

2.12.2. Preparation of Media Based on Fruit Residues

Orange residues were obtained from a fruit juice factory (BELSO, Ankara) and processed as described for sludge samples. The residues were dried at 80^{o} C till no wet particles remained. The dried bulk material was ground to a fine homogenous powder. The powder was stored at 4^{o} C until use. 10 to 20 g of fruit powder was suspended in dH_2O and the resulting suspension was filtered through a Whatman No.1 paper. The pH of the suspension was

adjusted to 7.2 and the resulting residue-based medium was next sterilized by autoclaving at 121^{o} C for 15 min.

2.12.3. Preparation of Media Based on Processed Broiler Litter

Samples of broiled litter were obtained from Yılmazer Tavukçuluk ve Yumurta Ltd. (Kütahya), dried at at 80^{o} C till no wet particles remained, ground to a fine homogenous powder and whenever indicated, processed as explained below.

2.12.3.1. Base Hydrolysis

The processing of broiler litter by base hydrolysis was adopted from Caldwell (2006) who treated cellulosic raw materials with base for a bioethanol process. The 10 to 20 g of dried broiler litter was soaked in 25 ml of %8 (w/v) NaOH and rapidly stirred constantly at 200^{0}C for 1 h. The resulting broiler litter suspension was cooled with dH_2O and filtered through Whatman No.1 filter paper. Its volume was made up to 1 L with dH_2O and the pH was adjusted to 7.2.. The resulting broiler-based media were ready to use after autoclaving at 121^{o} C for 15 min.

2.12.3.2. Acid Hydrolysis

The processing of broiler litter in strong acid was adopted from Dan Burden (2006, http://www.agmrc.org) who used acid treatment method for conversion of lignocellulosic materials into fermentable sugars. Acid hydrolysis was performed in five different ways:

I. Direct hydrolysis in 2N HCl at 50° C for 1 h:

10 to 20 g of dried broiler litter was suspended in 2N HCl which was then stirred at 50° C for 1 h. After cooling, the pH was adjusted to 7.2. The resulting broiler-based media were ready to use after autoclaving at 121° C for 15 min.

II. Direct hydrolysis in 2N HCl at room temperature, overnight:

As in (i), but hydrolysis was performed overnight at room temperature.

III. Direct hydrolysis in 1N HCl at 100^{0}C for 1h:

As in (i), but hydrolysis was performed in 1N HCl at 100^{0}C for 1h.

IV. Solid/liquid separation followed by hydrolysis of solids in 2N HCl at 50° C for 1 h:

10 to 20 g of dried broiler litter was suspended in 100 mL of dH_2O and filtered through a Whatman No.1 filter paper. The solids were collected and exposed to 5 ml of 2N HCl at 50° C for 1 h. After cooling, the suspension was filtered through Whatman No.1 filter paper. The liquid collected was mixed with the liquid obtained from the first filtration. Total volume was made up to 1 L with dH_2O and the pH was adjusted to 7.2. The resulting broiler-based media were ready to use after autoclaving at 121° C for 15 min.

V. Hydrolysis in 2% sulfuric acid at 50° C for 2 h:

As in (i), but 2% sulfuric acid instead of HCl was employed for the hydrolysis.

2.12.4. Inoculum Preparation

For inoculum preparation, the glycerol stock of each *Bt* strain was first inoculated (0.1%) into 50 mL of DSM in a 250 mL flask and cultured overnight by shaking at 30°C at 200 rpm. Unless otherwise indicated, DSM

cultures of the strains were used to inoculate (1%) 50 mL of low-cost media in 250 mL flasks and after an overnight growth, 1% aliquots were used as inocula to start fermentation in 50 mL of the respective, fresh low-cost media.

2.13. Analytical Procedures

2.13.1. Elemental Analysis

Chemical composition of both processed and unprocessed broiler litter was analyzed at Middle East Technical University Central Laboratory. For this, an elemental analyzer (LECO, CHNS-932) was used. The analyzer offers a rapid, simultaneous, multi-elemental determination of carbon, hydrogen, nitrogen, and sulfur in homogenous microsamples (2 milligrams). A representative sample of minimum 10 mg homogenous solid or liquid is required for analysis.

2.13.2. Trace Metal Analysis

Trace metal composition of both processed and unprocessed broiler litter was analyzed at Middle East Technical University Central Laboratory. Inductively Coupled Plasma (ICP) was the analytical technique used for multi-element detection. Sample requirements of trace metal analysis and above-mentioned element analysis are different. While there is no need to supply blank solution in element analysis, a blank should also be supplied with the sample in trace metal analysis if the sample is brought in solution.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Effects of Glucose on Lepidoptera-, Diptera- and Coleoptera-Specific Crystal Protein Biosynthesis

Fermentation and medium optimization is required for each particular *Bt* strain as different strains may have different responses to nutritional and cultural conditions (Kaur, 2000).

A comparison was made between the representative members of three different subspecies of *Bt* to observe toxin yield with respect to the nutritional conditions. Three different *Bt* subspecies were *Bt kurstaki* (strain 81), *Bt israelensis* (strain HD500) and *Bt tenebrionis* (strain 3203), producing lepidoptera- and diptera-specific Cry1 and Cry2, diptera-specific Cry4Ba and Cry11Aa and coleoptera-specific Cry3Aa toxins, respectively. Choosing a standard growth medium that favours growth and toxin biosynthesis in all these strains was the first attempt in this study before altering medium composition to improve production. Difco's Sporulation medium (DSM) previously used for *Bt tenebrionis* (Kurt et al., 2005b) was selected as a common cultivation medium as it fairly supported growth and toxin production for all strains (data not shown).

Toxin production was monitored at 24 h time intervals for 72 h in DSM medium, when the strains were grown at 30 ^{0}C by shaking at 200 rpm. The strains showed similar toxin production patterns throughout the incubation. The toxin yields in all strains were almost equally high in 24th and 48th h, and

except for Cry2 toxin, a general disappearance of toxins was recorded in 72nd h, representing aged cultures (Figure 3.1.a, b and c). In *Bt* 3203, 73 kDa Cry3Aa protoxin predominated at 24 h after which it was replaced by processed, 65 kDa toxin, as recorded earlier with another CryAa3 producing *Bt* strain (namely Mm2, a local isolate) indicating that the proteolysis was not complete till this time. Carroll et al. (1997) found predomination of protoxin in nutrient-rich broths and explained this as a manifestation of lower production of bacterial proteases in such conditions.

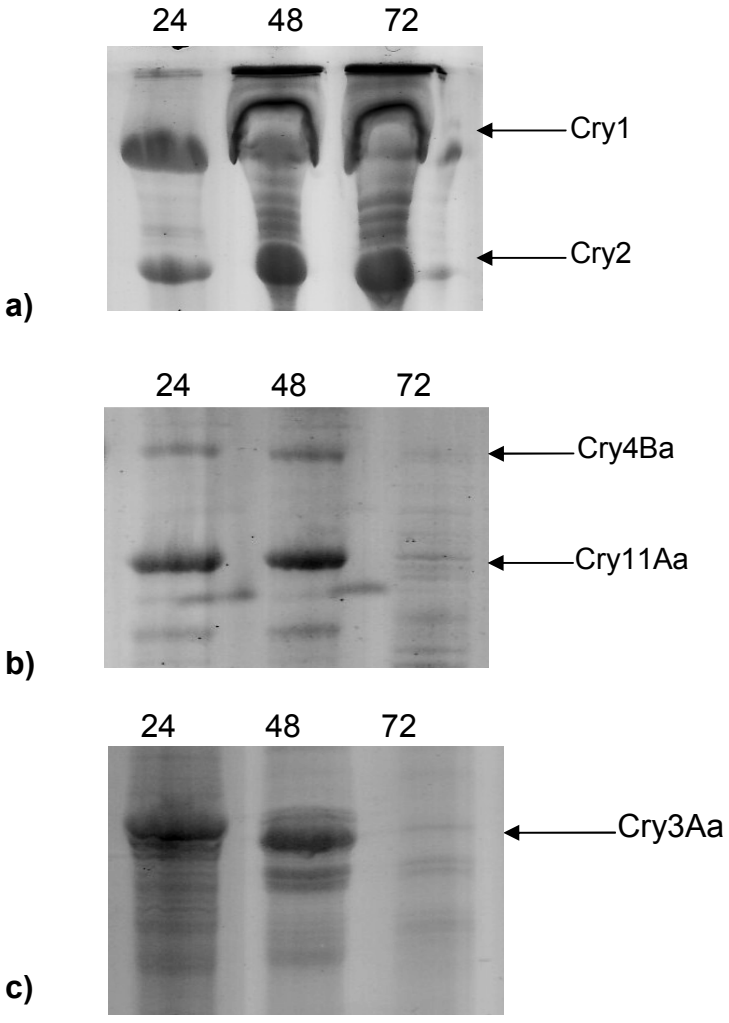


Figure 3. 1. Coomassie-stained SDS–PAGE showing Cry1 and Cry2 toxin biosynthesis by *Bt* 81 (a), Cry4Ba and Cry11Aa toxin biosynthesis by *Bt* HD500 (b) and Cry3Aa toxin biosynthesis by *Bt* 3203 (c) in DSM medium.

Regarding glucose and carbon sources effect, previous experiments with *Bt* 81 producing Cry1 and Cry2 and *Bt* HD500 producing Cry4Ba and Cry11Aa were conducted in our laboratory using a different medium, namely Yousten's Synthetic Medium (YSM). To investigate carbon source regulation of Cry1 and Cry2 toxin production, various carbohydrates were incorporated into YSM, each at a final concentration of 1 g/L (İçgen *et al*., 2002b) The highest cell densities and the lowest sporulation frequencies were obtained in media containing glucose or maltose. Toxin yields on these sugars were markedly less than those obtained on lactose, sucrose and inulin which were not good carbon sources for growth, but supported the synthesis of a large amount of crystal protein as well as high sporulation frequencies. Whey and molasses, which can be used as low-cost and readily available substrates at an industrial scale, were stimulatory for crystal protein formation and sporulation, but were poor for growth. The finding that certain carbon sources (di- and polysaccharides) are preferred over glucose suggested a "glucose effect" operating in crystal protein synthesis. On the other hand, the use of maltose, starch and dextrin in place of glucose did not improve crystal titers. Regarding the close association between crystal protein formation and sporulation (Afkhami *et al*., 1993; Özcengiz *et al*., 1990), regulation by carbon sources could be regarded as either a direct control of crystal protein formation or an indirect effect resulting from stimulation or suppression of sporulation. Crystal protein synthesis did not seem to be solely a manifestation of sporulation since growth on starch and dextrin yielded reasonable levels of spores, but relatively little toxin.

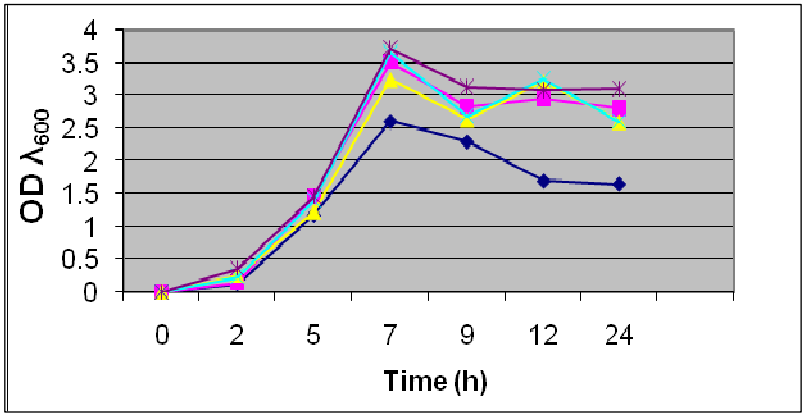
As to the carbon source effect on Cry4Ba and Cry11Aa biosynthesis in *Bt* HD500, poor toxin production in glucose medium was evident also for these types of toxins. Not only glucose, but also starch and molasses were repressive and/or inhibitory for toxin production, blocking in particular the formation of the 134 kDa Cry4Ba component (Özkan *et al*., 2003). In contrast, dextrin, whey, maltose, lactose, inulin, glycerol and sucrose

influenced the toxin biosynthesis in a positive manner and supported good growth. Sporulation was poor in maltose medium as compared to the others; with lactose or dextrin, on the other hand, the sporulation efficiency was maximal.

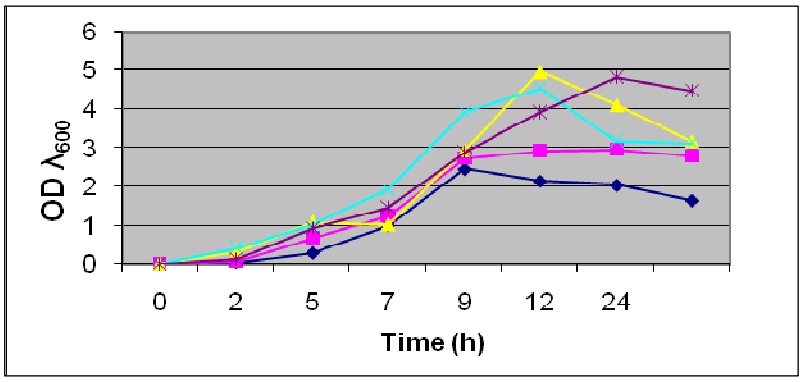
The effect of increasing concentrations of glucose had not been investigated in above-mentioned studies. In a more recent study, the possibility of catabolite repression of Cry 3Aa toxin synthesis was examined by running parallel cultures of *Bt* MM2 in DSM with 0.5, 5 and 10 g /L concentrations of glucose or sucrose, respectively (Kurt et al., 2005b). Although relative toxin yields in these media did not suggest a glucose effect, the results of the resuspension experiments implied suppression of toxin synthesis by increasing concentrations of glucose, thus a contradiction remained.

In the present study, the effects of glucose concentration in DSM medium on growth, sporulation and toxin biosynthesis by different types of Cry producers were studied in the same set of experiment. Figure 3.2 and Figure 3.3 show growth curves and sporulation frequencies, respectively, for *Bt* 81, *Bt* HD500 and *Bt* 3203 when cultivated for 24 h in DSM medium with different glucose concentrations in a range of 0 to 10 g/L. As expected, increasing concentrations of glucose stimulated the growth of all strains, while gradually lowering sporulation.

a)



b)



c)

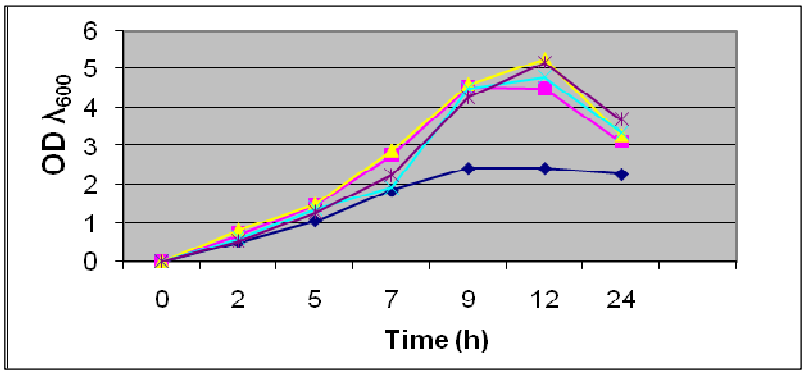


Figure 3. 2. Growth of *Bt* 81 (a), *Bt* HD500 (b) and *Bt* 3203 (c) in DSM with different glucose concentrations of (♦) 0 g/L, (■) 2.5 g/L, (▲) 5 g/L, (x) 7.5 g/L, and (ж) 10 g/L.

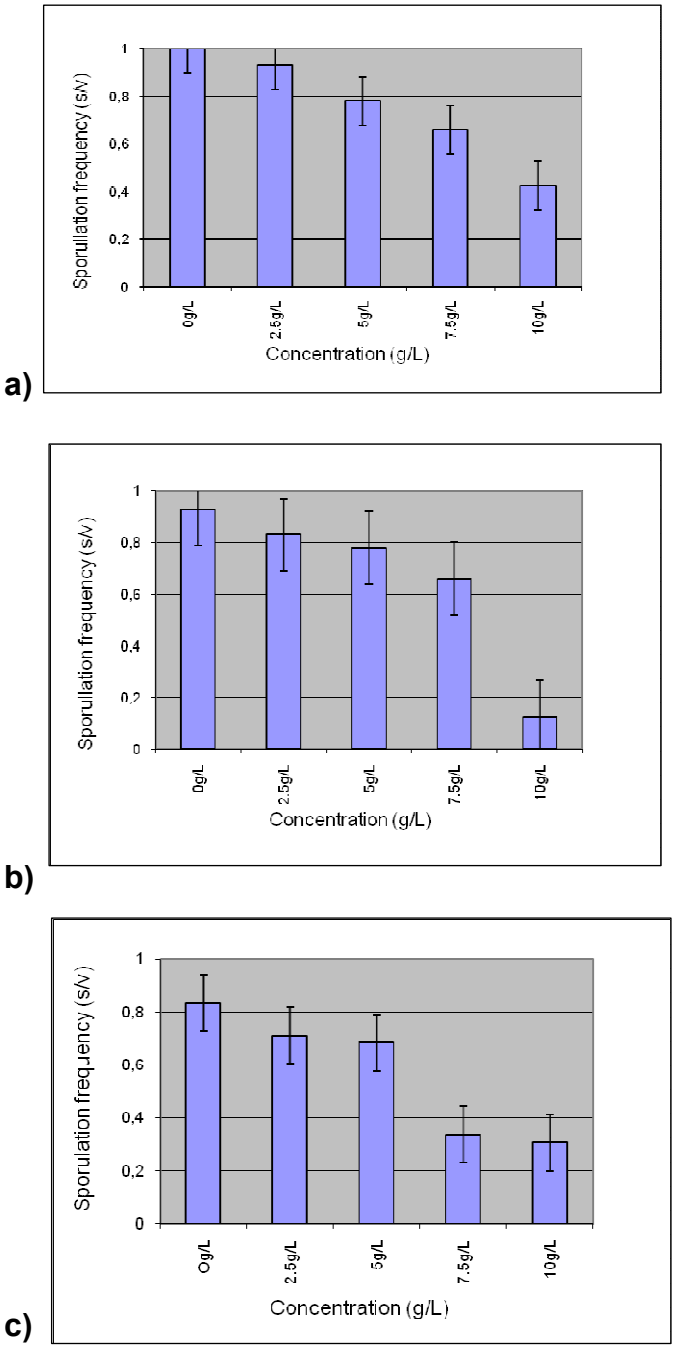


Figure 3. 3. Sporulation frequency (S/V) of *Bt* 81 (a), *Bt* HD500 (b) and *Bt* 3203 (c) when grown in DSM with different glucose concentrations.

Highest Cry1 and Cry2 production in *Bt* 81 occurred in a medium either lacking glucose or having a low glucose concentration of 2.5 g/L till 24th h. However, in 48 h cultures, the levels of these toxins were almost equal in a wide range of 0 to 7.5 g/L glucose. 10 g/L glucose suppressed the production of toxin biosynthesis in this *Bt* strain (Figure 3.4.a). Similar to the Cry1 and Cry2 toxins, the highest levels of Cry4Ba and Cry11Aa production in *Bt* HD500 occurred in a medium lacking glucose at 24th h while a moderate glucose concentration of 5 g/L favored toxin biosynthesis at 48th. As in the case of *Bt* 81, 10 g/L glucose suppressed the production of toxin biosynthesis in *Bt* HD500 (Figure 3.4.b). Cry3a production by *Bt* 3203, on the other hand, was the best on 5 g/L glucose at 24th h which became equally good on 2.5 and 5 g/L glucose at 48th h (Figure 3.4.c). Taken together, for all *Bt* strains, a glucose concentration of 5 g/L generally yielded high levels of bioinsecticidal toxins at 48 h fermentation.

Among the strains compared in the present study, general suppression of toxin yields by a high glucose concentration of 10 g/L indicated that carbon catabolite repression ("glucose effect") typical for secondary metabolism (Demain, 1995) is a general phenomenon applying to the biosynthesis of different classes and types of crystal protein.

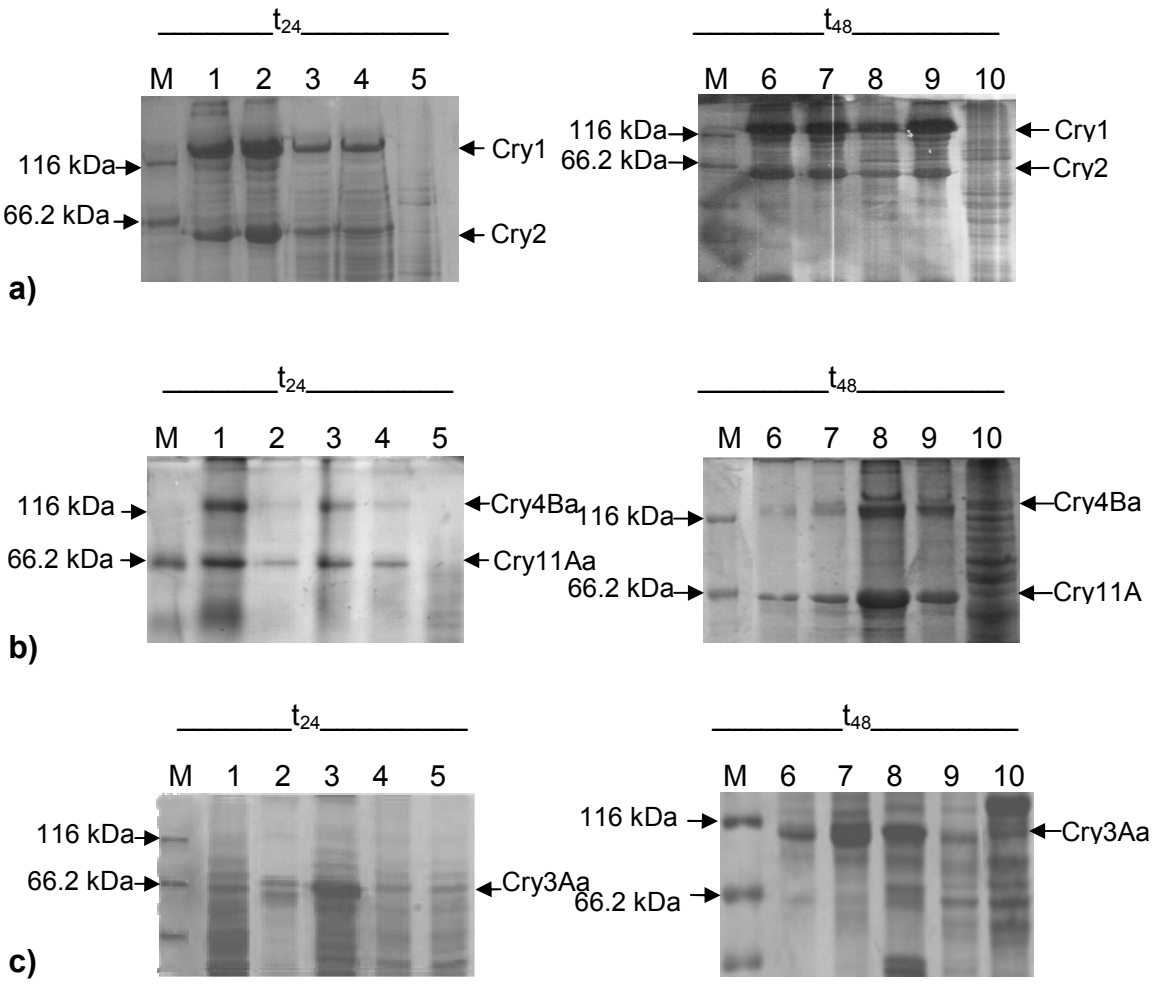


Figure 3. 4. Effects of glucose concentration on Cry1 and Cry2 (a), Cry4Ba and Cry11Aa (b), and Cry3Aa (c) biosynthesis at 24 h and 48 h. M, Molecular weight markers. Glucose concentrations in Lanes 1 and 6: 0 g/L; Lanes 2 and 7: 2.5g/L; Lanes 3 and 8: 5 g/L; Lanes 4 and 9: 7.5g/L and Lanes 5 and 10: 10g/L.

3.2. Effects of Inorganic Phosphate on Lepidoptera-, Diptera- and Coleoptera-Specific Crystal Protein Biosynthesis

Phosphate is considered to be essential for protein expression and also critical for cellular growth (Yang and Wang, 2000). Recently, inorganic phosphate was found to be the most crucial nutrient for Cry3Aa production, causing a drastic increase in toxin production when supplied at 200 mM (Kurt

et al, 2005b). The generality of the crucial role of Pi on Cry3Aa toxin synthesis was next demonstrated by using two other Cry3Aa producers, *Bt* subsp. *morrisoni* 1925 and 3123. The results suggested that Pi effect is common to different producers of the same toxin. The positive influence of high Pi concentration on crystal protein production had also been documented for two other *Bt* subspecies in our previous reports (Içgen et al. 2002b; Özkan et al. 2003). Nonetheless, YSM was used as the growth medium in these studies. Optimum concentration ranged between 50 and 100 mM for the synthesis of Cry1, Cry2, Cry4Ba and Cry11a toxins, 150 mM being inhibitory for Cry1 and Cry2. Moreover, the magnitude of the positive effect was much lesser in those cases.

To reveal the effects of inorganic phosphate (Pi) in DSM, three different *Bt* strains were grown in parallel DSM containing Pi at a concentration of 50 mM (as contained in DSM) and 200 mM, respectively. As shown in Figure 3.5., Cry1 and Cry2 levels in *Bt* 81 were almost equally high in the presence of 50 mM and 200 mM levels of Pi. However, biosynhesis of Cry4Ba and Cry11Aa was negatively influenced especially at early stages of fermentation when Pi concentration was raised to 200 mM. Contrarily, except for 24^{th} h, Cry3Aa levels significantly increased when Pi concentration was increased to 200 mM. It was concluded that inorganic phosphate level has a great impact on Cry3Aa biosynthesis and slight impact on Cry4Ba, Cry11Aa while Cry1 and Cry2 production was not responsive to high Pi. As to the Pi effect on Cry3Aa biosynthesis, our findings further supported the postulation of Kurt, *et al* (2005b) about the generality of Pi effect on Cry3Aa biosynthesis as we revealed the same Pi effect with a different Cry3Aa producer strain in a different medium.

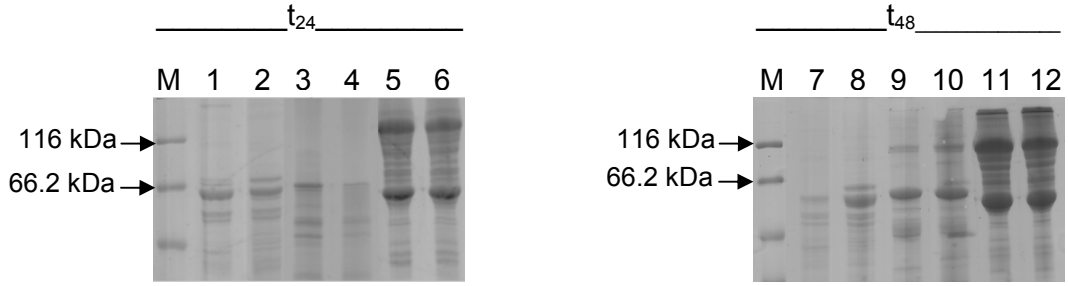


Figure 3. 5. Coomassie-stained SDS–PAGE showing the effect of Pi concentration on Cry3Aa (Lanes 1, 2, 7, 8, 13 and 14), Cry4Ba and Cry11Aa (Lanes 3, 4, 9, 10, 15 and 16) and Cry1 and Cry2 (Lanes 5, 6, 11, 12, 17 and 18) biosynthesis at 24th, 48th. M, molecular weight markers. Toxin levels on DSM with 50mM Pi are shown on lanes 1, 3, 5, 7, 9 and 11 whereas those on DSM with 200mM are shown on lanes 2, 4, 6, 8, 10 and 12.

3.3. Studies to Define a Low-Cost Medium for Lepidoptera-, Diptera- and Coleoptera-Specific Crystal Protein Biosynthesis

Although *Bt*-based formulations are widely used in pest control programs, the large scale production of the organism is expensive because of the high cost of the medium. Effects of low-cost media supplements such as fodder yeast and agro-industrial by-products (Salama et al., 1983), legume seeds and dried cow blood (Obeta and Okafor 1984), gruel and fishmeal (Zouari et al. 2002), wheat bran (Vimala Devi et al. 2005), wastewater sludge (Lachhab et al. 2001; Vidyarthi et al. 2002) and re-use of culture supernatant (Luna et al. 2004) were studied for anti-lepidopteran and anti-dipteran toxin production. On the other hand, except for a solid-state fermentation using broiler litter (Adams et al. 2002), there appears no report on production of Cry3Aa anti-coleopteran toxin by using less well-defined, inexpensive and commercially realistic media. In the present study, we attempted to develop a cost-effective medium, based on inexpensive, locally available raw materials including wastewater sludge, broiler and fruit residues and to determine toxin production capacities of our strains

Wastewater sludge (containing carbon, nitrogen, phosphorus and other nutrients) supports growth and sporulation of *Bt*. However, the results obtained so far indicated low concentration of viable cell-spores, low entomotoxicity and poor specific growth rate. Many factors (oxygen concentration, pH, medium composition, sludge nature, sludge solids concentration and inoculum) can influence the *Bt* production process (Lachhab *et al*., 2001; Vidyarthi *et al*., 2002)

Sludge bears mixed cultures of microorganisms. Microorganismal activity is highly important and one of the major causes affecting nutritional value of sludge. The nutritional value of the sludge changing enormously while the feed of the broiler and the temperature of the flock feeding house is constant so as the conditions of fruit juice factory. On the other hand, the temperature of the wastewater treatment plants always change as such plants are exposed to open air and the nutritional value of the sludges varies with respect to temperature.

3.3.1. Wastewater Sludge as a Raw Substrate

By using two different wastewater sludge samples (sludge sample 1 and 2 which were taken in December 2006 and April 2007, respectively), sludge-based media were prepared as described in Section 2.12.1. Sludge solids concentration was first optimized by cultivating the strains for 24 h on media with sludge solids concentrations (SSCs) of 10, 15, 20 and 25 g/L, respectively, for both sludge sample 1 and 2. Neither could support the growth of *Bt* 3203 at the SSCs tested (data not shown). A SSC of 15 g/L resulted in best toxin yields for *Bt* 81 and/or *Bt* HD500 (Figure 3.6) and higher concentrations were inhibitory (data not shown). Although *Bt* 81 could not grow on sludge sample 2, it gave promising toxin yields on sludge sample 1. As to *Bt* HD500, Cry11Aa yield was found to be higher on sludge sample 2

as compared to that on sludge sample 1 while Cry4Ba toxin yield did not much vary (Figure 3.6).

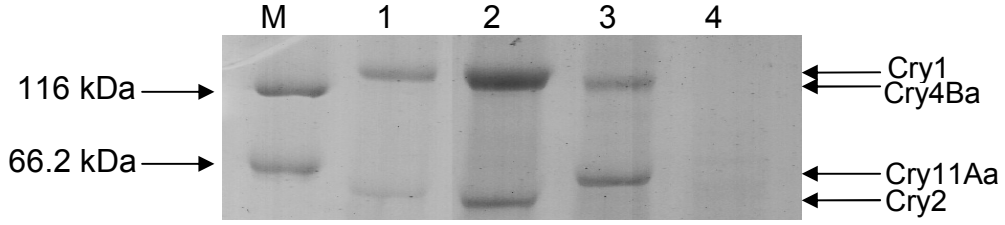


Figure 3. 6. Coomassie stained SDS-PAGE of crystal proteins produced on two different sludge samples each with a SSC of 15 g/L. M: Molecular weight markers, Lane1: *Bt* HD500 in sludge sample 1, Lane 2: *Bt* 81 on sludge sample 1, Lane3: *Bt* HD500 on sludge sample 2, Lane 4: *Bt* 81 on sludge sample 2.

As based on these results, it was concluded that a wastewater sludge-based medium can be convenient for the cultivation of *Bt* 81 and possibly other producers of Cry1 and Cry2 toxins. Still, the seasonal variability of the chemical composition of wastewater sludge must be taken into consideration.

According to some literature reports, suspended solids were removed from raw media by either centrifugation or filtration (Brar *et al*., 2005), and in certain cases, an additional source of nutrients was also required to improve growth and sporulation of *Bt* (Lachhab et al., 2001). Therefore, we next attempted to investigate the effect of (i) filtration of sludge suspension for removal of large solid particles and (ii) supplementation of sludge suspension with nutrients (addition of 1 g/L Nutrient broth) on toxin production by *Bt* 81. A control culture was grown on DSM in order to be able to correlate toxin yields to those that can be obtained in standard medium. As can be seen in Figure 3.7, neither filtration, nor nutrient supplementation did have a contribution to toxin yields for sludge sample 1 while toxin formation could occur only in filtered and nutrient supplemented medium based on sludge sample 2. This can be possibly due to low nutrient concentration of the latter sample, still our

experiments did not confirm beneficial effects of filtration or nutrient supplementation.

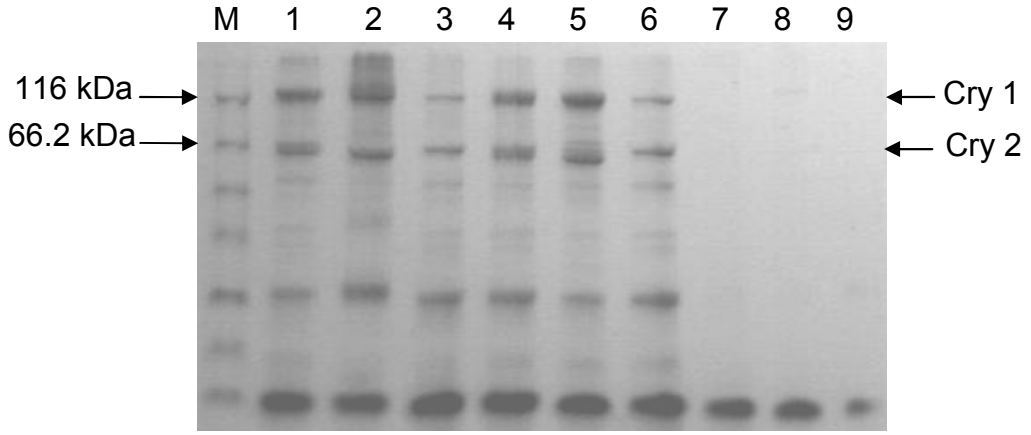


Figure 3. 7. Coomassie stained SDS-PAGE of crystal proteins produced by *Bt* 81 on different sludge-based media each with a SSC of 15 g/L. M: Molecular weight markers, Lane 1: DSM (control); Lane 2: Sludge media based on sample 1, filtered, Nutrient broth (0.1%) added; Lane 3: Sludge media based on sample 1, not filtered, Nutrient broth (0.1%) added; Lane 4: Sludge media based on sample 1, filtered, no Nutrient broth (0.1%) added; Lane 5: Sludge media based on sample 1, not filtered, no Nutrient broth (0.1%) added; Lane 6: Sludge media based on sample 2, filtered, Nutrient broth (0.1%) added; Lane 7: Sludge media based on sample 2, not filtered, Nutrient broth (0.1%) added; Lane 8: Sludge media based on sample 2, filtered, no Nutrient broth (0.1%) added; Lane 9: Sludge media based on sample 2, not filtered, no Nutrient broth (0.1%) added. Arrow is indicating Cry1 and Diamond is indicating Cry2.

3.3.2. Fruit Residues as a Raw Substrate

To search for the possibility of the use of fruit residues as a suitable low-cost raw substrate for *Bt*-based bioinsecticide production, fruit residue-based media were prepared as described in section 2.12.2. Solid concentration in these media was kept as 15% (w/v). The use of two types of inoculum was aimed at, DSM-grown inoculum and fruit residue-grown inoculum. However, since almost no growth occurred on fruit residues, fruit residue-based media was supplemented with Nutrient broth at a final concentration of 1 g/L. Toxin

protein biosynthesis at 24th h, 48th h and 72nd h of fermentation are shown in Figure 3.8, 3.9 and 3.10, Lanes 5, 6 and 7, respectively. 50mM Pi and 200 mM Pi containing DSM media (Lanes 1 and 2) were used as positive controls in the experiment. Toxin formation on fruit residue-based media was detectable only after 48 h fermentation, Cry2, Cry3Aa and Cry11a being the toxins formed at very low levels. It was concluded that a fruit residues, even when supplemented with Nutrient broth, do not constitute a suitable medium for *Bt* fermentations.

3.3.3. Broiler Litter as a Raw Substrate

To search for the possibility of the use of broiler litter as a suitable low-cost raw substrate for *Bt*-based bioinsecticide production, broiler litter-based media were prepared as described in section 2.12.3. Solid concentrations in these media were kept as 15% (w/v). Two types of inoculum were prepared for the experiments, DSM-grown inoculum and broiler litter-grown inoculum, prepared as indicated in section 2.12.4. The cell concentration attained in DSM-grown seed culture was 2.5x10^{7}/ml CFU/mL, while broiler litter contained 5x10^{5} CFU/mL. Toxin protein biosynthesis at 24th h, 48th h and 72nd h of fermentation are shown in Figure 3.8, 3.9 and 3.10. The 50mM Pi and 200 mM Pi containing DSM were the positive controls in the same set of experiment. Broiler litter seemed to be a much better substrate than fruit residues in that except for Cry4Ba, some degree of production of each toxin was observed at almost every stage of fermentation. Although the inoculum type influenced the strains in a different way at different stages of fermentation, DSM-grown inoculum generally worked better for broiler litter-based medium.

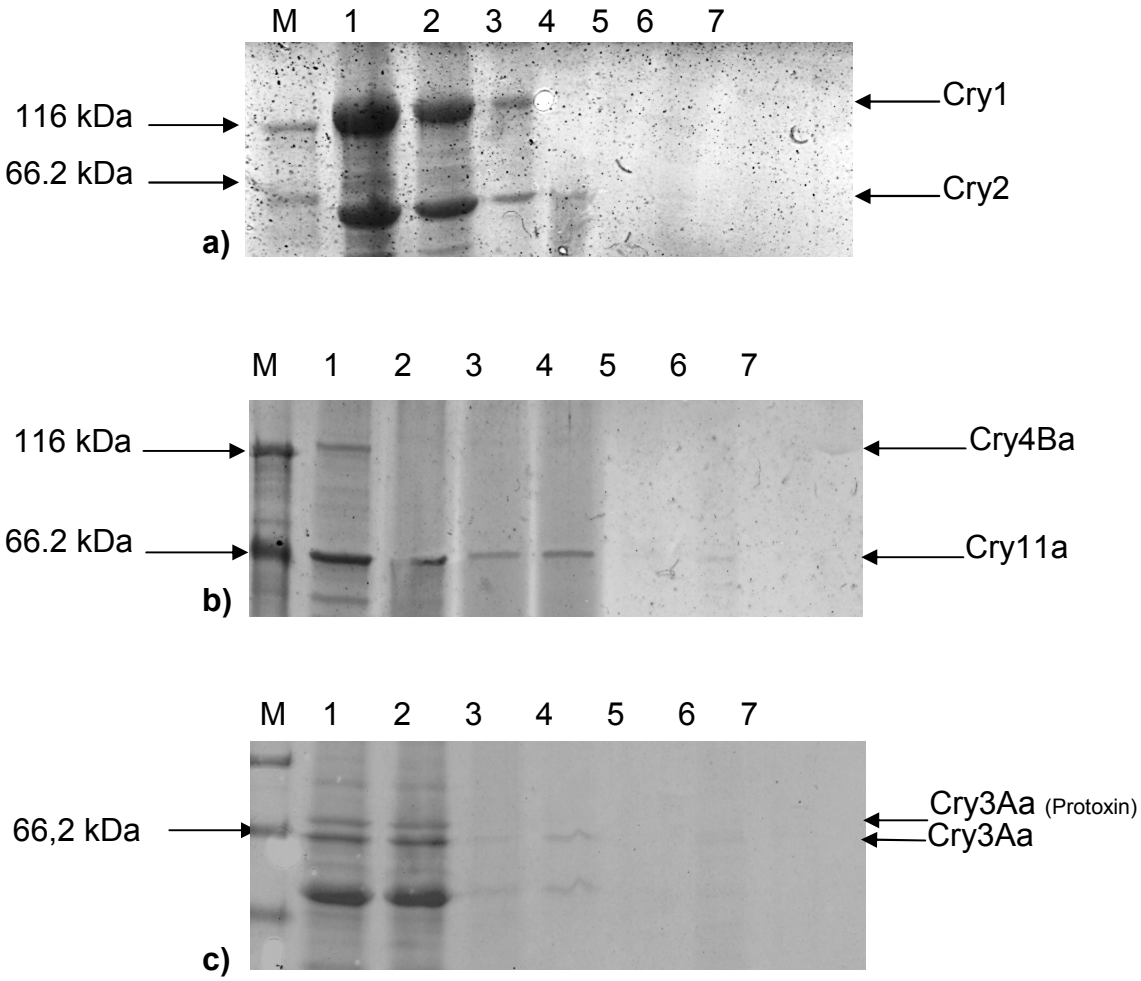


Figure 3. 8. Coomassie-stained SDS–PAGE showing the biosynthesis of Cry1 and Cry biosynthesis by *Bt* 81 (a), Cry4Ba and Cry11a biosynthesis by *Bt* HD500 (b) and Cry3Aa biosynthesis by *Bt* 3203 (c) after 24 h incubation. M: Protein molecular weight markers. Lane 1: DSM control (50 mM Pi); Lane 2: DSM control (200 mM Pi); Lane 3: Broiler litter-based medium, inoculum prepared in the same medium; Lane 4: Broiler litter-based medium, DSM-grown inoculum; Lane 5: Fruit residues, DSM-grown inoculum; Lane 6: Fruit residues supplemented with Nutrient broth (1 g/L), DSM-grown inoculum; Lane 7: Fruit residues supplemented with Nutrient broth (1 g/L), inoculum prepared in the same medium.

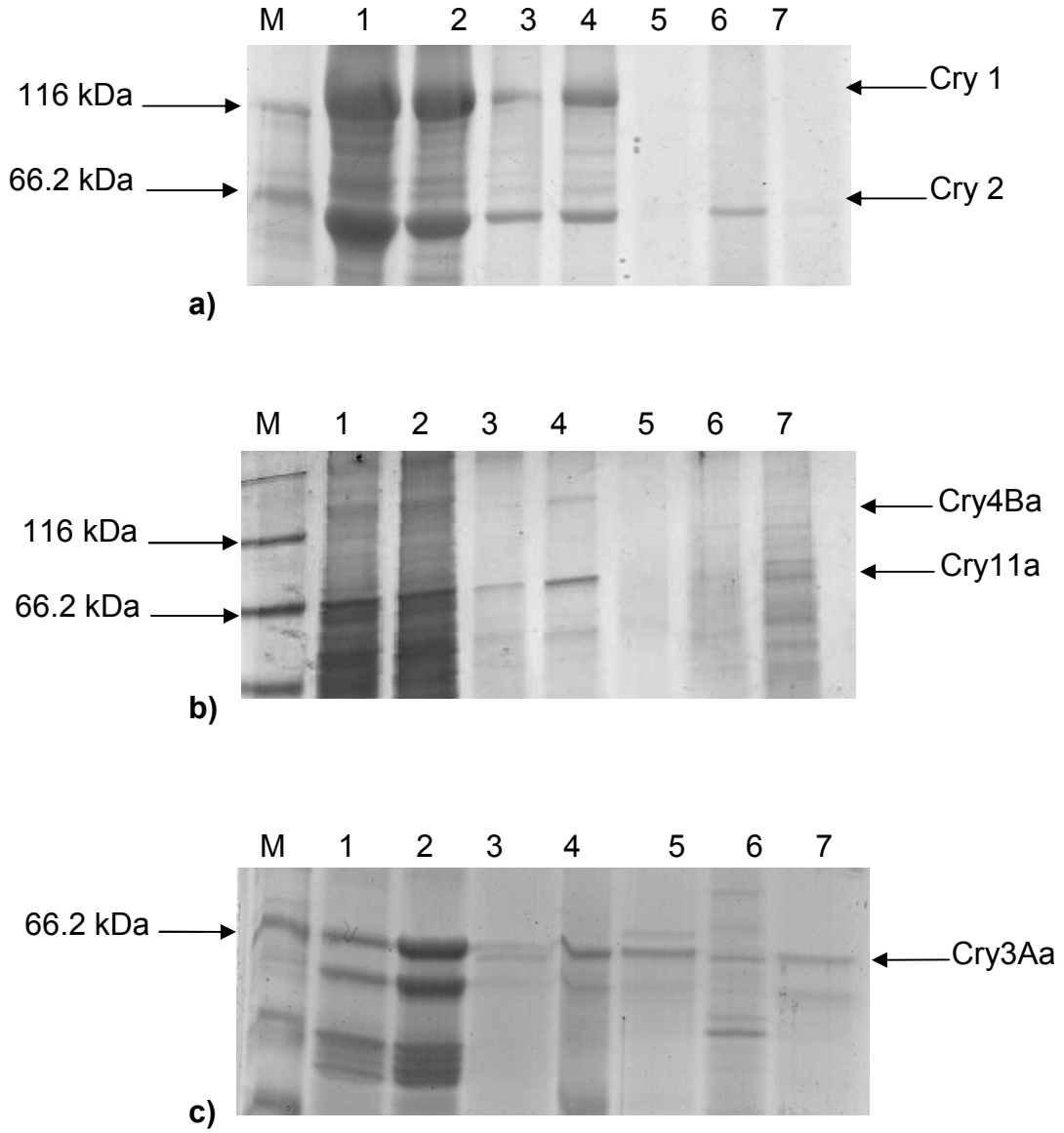


Figure 3. 9. Coomassie-stained SDS–PAGE showing the biosynthesis of Cry1 and Cry biosynthesis by *Bt* 81 (a), Cry4Ba and Cry11a biosynthesis of *Bt* HD500 (b) and Cry3Aa biosynthesis of *Bt* 3203 (c) after 48 h incubation. M: Protein molecular weight markers. Lane 1: DSM control (50 mM Pi); Lane 2: DSM control (200 mM Pi); Lane 3: Broiler litter-based medium, inoculum prepared in the same medium; Lane 4: Broiler litter-based medium, DSM-grown inoculum; Lane 5: Fruit residues, DSM-grown inoculum; Lane 6: Fruit residues supplemented with Nutrient broth (1 g/L), DSM-grown inoculum; Lane 7: Fruit residues supplemented with Nutrient broth (1 g/L), inoculum prepared in the same medium.

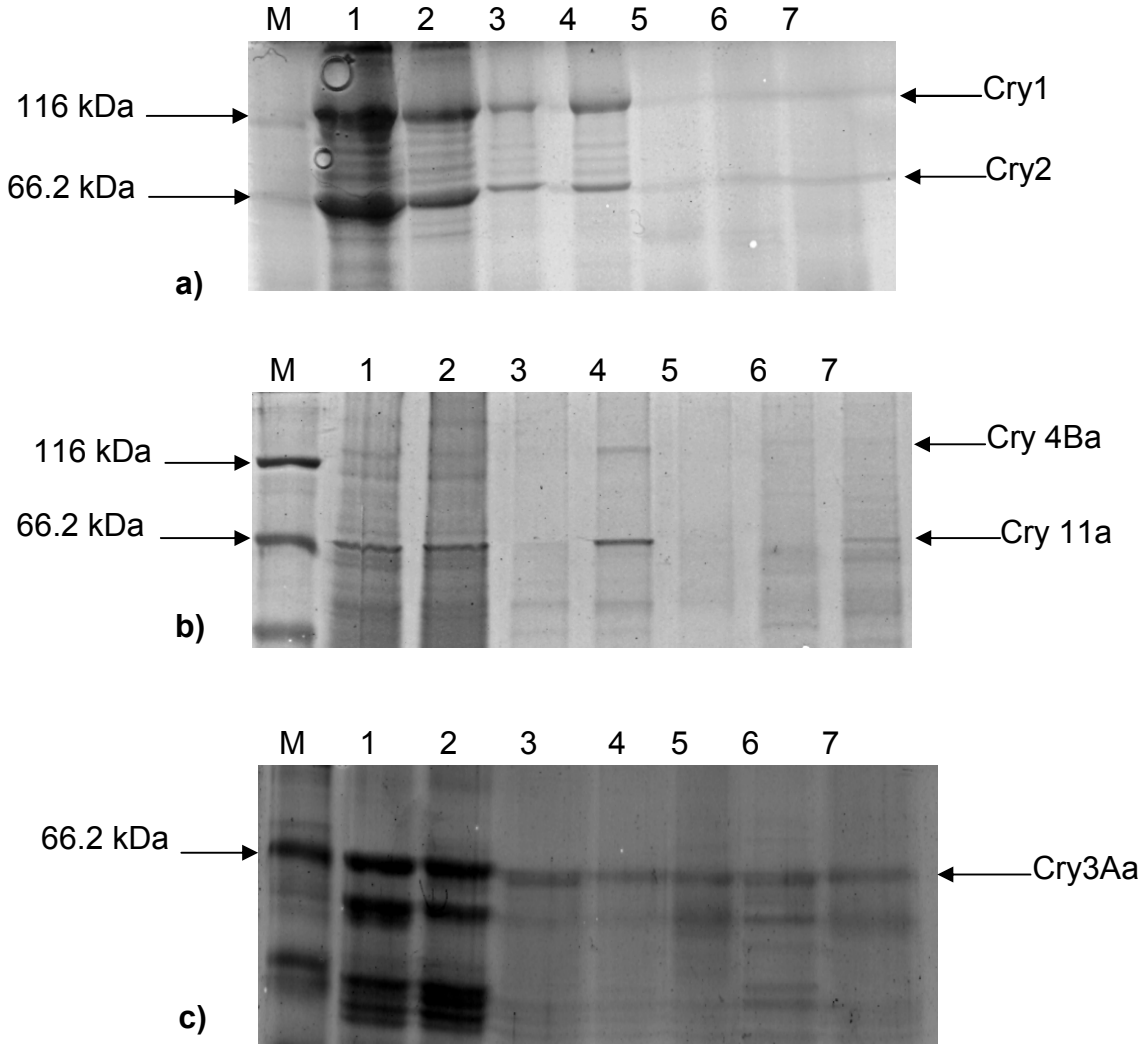


Figure 3. 10. Coomassie-stained SDS–PAGE showing the biosynthesis of Cry1 and Cry biosynthesis by *Bt* 81 (a), Cry4Ba and Cry11a biosynthesis of *Bt* HD500 (b) and Cry3Aa biosynthesis of *Bt* 3203 (c) after 72 h incubation. M: Protein molecular weight markers. Lane 1: DSM control (50 mM Pi); Lane 2: DSM control (200 mM Pi); Lane 3: Broiler litter-based medium, inoculum prepared in the same medium; Lane 4: Broiler litter-based medium, DSM-grown inoculum; Lane 5: Fruit residues, DSM-grown inoculum; Lane 6: Fruit residues supplemented with Nutrient broth (1 g/L), DSM-grown inoculum; Lane 7: Fruit residues supplemented with Nutrient broth (1 g/L), inoculum prepared in the same medium.

The elementary composition of flock manure is greatly affected by the feeding types and the use of antimicrobial agents in flock feed (Adams *et al*. 2002). Another factor affecting its quality is related with its storage. The nutritious value of the flock litter rapidly decreases by time upon exposure to air. The litter should be rapidly processed and carefully handled in storage space. Pretreatment is essential for bioconversion of most lignocellulosic materials. Pretreatment alternatives include mechanical size reduction, heat, steam, steam explosion, autohydrolysis, acid hydrolysis, enzyme treatment, alkali treatment, ammonia, chemical pulping, solvent extraction, and various combinations of these processes (www.agmrc.org). Since toxin yields were shown to be promising on broiler litter, the litter was subjected to acid and base pretreatment with the aim of hydrolyzing polymeric material, hence improving its nutritional value as a complex substrate.

Since *Bt* 81 was the best Cry toxin producer on broiler litter, this strain was employed for further experiments which involved the use of processed broiler litter. As indicated in section 2.12.3, base hydrolysis and acid hydrolysis were the techniques used for substrate pre-treatment. In the case of acid hydrolysis, five alternative pretreatments were made: I. 2N HCl at 50^0C for 1 h; II. 1N HCl at 100^0C for 1 h; III. 2N HCl at room temperature, overnight; IV: solid/liquid separation followed by hydrolysis of solids in 2N HCl at 50^o C for 1 h; and V. 2% sulfuric acid at 50^o C for 2 h. Our results showed that base hydrolysis was a more efficient pre-treatment than direct acid hydrolyses (pretreatments I, II, III and V), however filtration of broiler litter suspension followed by acid hydrolyses of solids (pretreatment IV) resulted in highest toxin yields (Figure 3.11). Therefore, pretreatment IV was used for broiler litter-based media preparation in the rest of the study and the term “processed broiler litter” referred to the media prepared with the substrate processed in this way.

Cry1 and Cry2 biosynthesis by *Bt* 81 as a function of processed broiler litter concentration after 24 h fermentation is shown in Figure 3.12. Although the toxin levels did not show striking differences at the substrate concentrations studied, a concentration of 15 g/L seemed to be optimal.

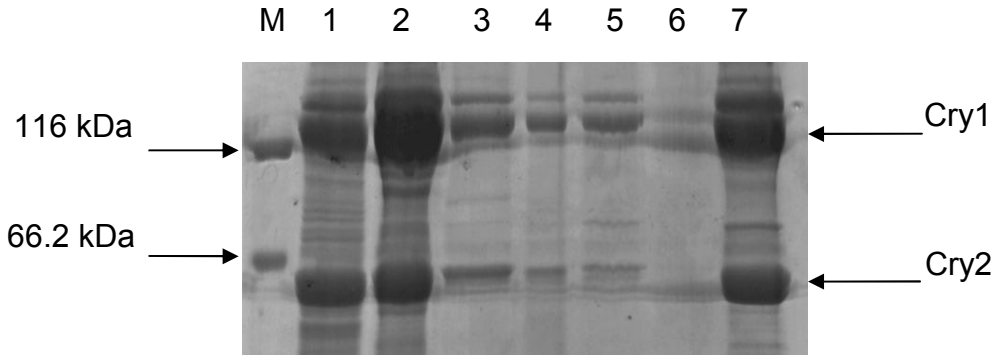


Figure 3. 11. Coomassie-stained SDS–PAGE showing Cry1 and Cry2 biosynthesis by *Bt* 81 on different broiler litter-based media when DSM-grown inocula were used. M: Protein molecular weight markers. Lane 1: Alkali-hydrolyzed; Lane 2: Acid-hydrolyzed, treatment IV; Lane 3: Acid-hydrolyzed, treatment I; Lane 4: Acid-hydrolyzed, treatment II; Lane 5: Acid-hydrolyzed, treatment III; Lane 6: Acid-hydrolyzed, treatment V; Lane 7: DSM control (50 mM Pi).

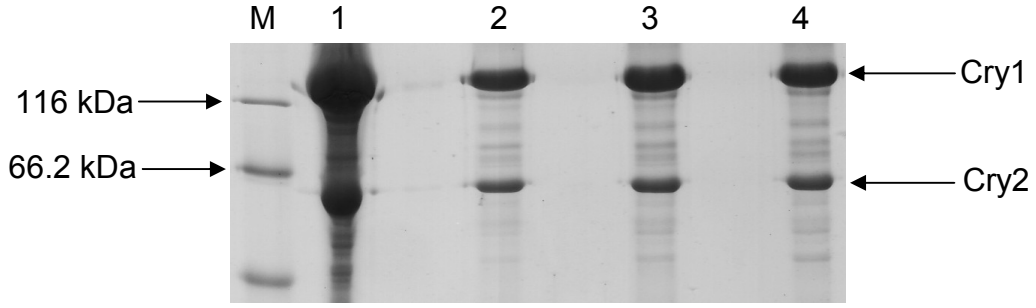


Figure 3. 12. Coomassie-stained SDS–PAGE showing Cry1 and Cry2 biosynthesis by *Bt* 81 as a function of processed broiler litter concentration after 24 h fermentation. M: Protein molecular weight markers. Lane 1: DSM control (50 mM Pi). Broiler solid concentrations of 10, 15 and 20 g/L are shown on Lane 2, 3 and 4, respectively.

It is known that during acid hydrolysis, ammonia and amino acids from hydrolyzed protein can react with mono-sugars in the hydrolyzed solution

under the high-temperature acidic conditions and can ultimately influence the final sugar yield (Fennema, 1996). Because of this reason, Liao *et al*. (2004) pretreated dairy manure by washing it three separate times with water and separating out the solids using a centrifuge. These washings were reported to be enough to cut the nitrogen content of the manure by half, and the resulting manure was much more qualified as a substrate for various industrial fermentations. In our study, the pretreatment IV which involved separation of solids from broiler litter suspension before acid hydrolysis most possibly had a beneficial effect by ensuring removal of unwanted. ammonia and amino acids from hydrolysis mixture.

Compounds exist in broiler litter that may inhibit growth of some microorganisms. For example, salinomycin, monensin and lasalocit are in feed rations as growth promoters and as coccidiostats (Asukabe *et al*., 1994). When broiler litter was used by Adams *et al*. (2002) in a solid state fermentation for production of biocontrol agents *Bt* and *Pseudomonas fluorescens*, it was subjected to extraction at concentrations of 50 g/L and 200 g/L with 90% (v/v) aqueous methanol. This extraction was reported to greatly reduce monensin content of broiler and improve cell and spore yields. In the present study, methanol extraction was not made. The company from which the broiler litter samples were obtained has been manufacturing poultry feed too (from whole corn plant and sugar beet leaves), and the authorities of the company guaranteed that they do not incorporate any antimicrobials to the feed, as they do not find much economical.

Although highly variable, one study found litter to have an average moisture content of 25% (wet basis) and contained 1.7% nitrogen, 0.81% phosphorus , and 1.25% potassium by weight (Raymond, 1974). We had to have our own elemental analysis made not only because the poultry management in Turkey might differ from worldwide sophisticated poultry management, but

also in order to be able to compare unprocessed and processed litter. Carbon and nitrogen contents as well as the trace elements contents of processed and unprocessed broiler litter are tabulated in Table 3.1 and 3.2, respectively.

Table 3. 1. Carbon and nitrogen contents and C:N ratios of processed and unprocessed broiler litter.

	Processed broiler litter	Unprocessed broiler litter
C	0,77 g/L	0.72 g/L
N	0,45g/L	1.14 g/L
C:N ratio	1.73	0.63

Table 3. 2. Trace elements contents of DSM, processed and unprocessed broiler litter.

	DSM (mg/L)	Processed broiler litter (mg/L)	Unprocessed broiler litter (mg/L)
Fe	0.56	1.03	1.19
Ca	20.04	77.52	85.13
Mg	12.31	16.10	14.95
Mn	0.55	0.68	0.67

There were only slight differences between the trace element contents of processed and unprocessed broiler litter. Their C and N contents, hence C:N ratios formed the only striking difference between them. C:N ratio of unprocessed broiler litter was considerably lower than that of the processed broiler litter. Elemental analysis also proved that pretreatment IV ensure a significant reduction in nitrogen content of the litter (by half). Concerning the effect of C:N ratio on delta-endotoxin formation, the study conducted by

Farrera *et al.* (1998) showed that a ratio of 7:1 improved the production of crystal protein Cry1Ac by *Bt* spp. *kurstaki* HD73. The methanol-extracted broiler litter with a C:N ratio of 8.5 gave much higher sporulation frequency when compared to C:N ratios of 13.5, 12.7 and 9.5 (Adams *et al*., 2002).

In order to find out an ideal C:N ratio and further improve Cry1 and Cry2 toxin yields on processed broiled litter, a new experiment was designed in which processed broiler litter-based media with different C:N ratios were compared. Knowing the C:N ratio processed broiled litter (Table 3.1), C:N ratios of parallel media were adjusted to 4, 6, 8 and 10, respectively, by adding appropriate amounts of glucose into each. To note, the maximum amount of glucose added was 3.68 g/L to attain the highest C:N ratio of 10. A C:N ratio of 4 was ideal for Cry1 and Cry2 production by *Bt* 81 as well as Cry4Ba and Cry11Aa biosynthesis by *Bt* HD500 (Figure 3.13.a and b). For Cry 3Aa toxin biosynthesis, on the other hand, C:N ratio was found to be a very important parameter as C:N ratios higher than 4 decreased delta-endotoxin yield remarkably (Figure 3.13.c.). There were also two other interesting findings. One was related with the type of inoculum. The use of DSM-grown inoculum or processed broiler litter-grown inoculum did not affect toxin production in *Bt* 81 and *Bt* HD500 while the inoculum grown on processed broiler litter performed definitely better in the case of *Bt* 3203. The other thing was that the great increase in toxin yields upon substrate processing as revealed for *Bt* 81 and *Bt* 3203 could not be seen with *Bt* HD500.

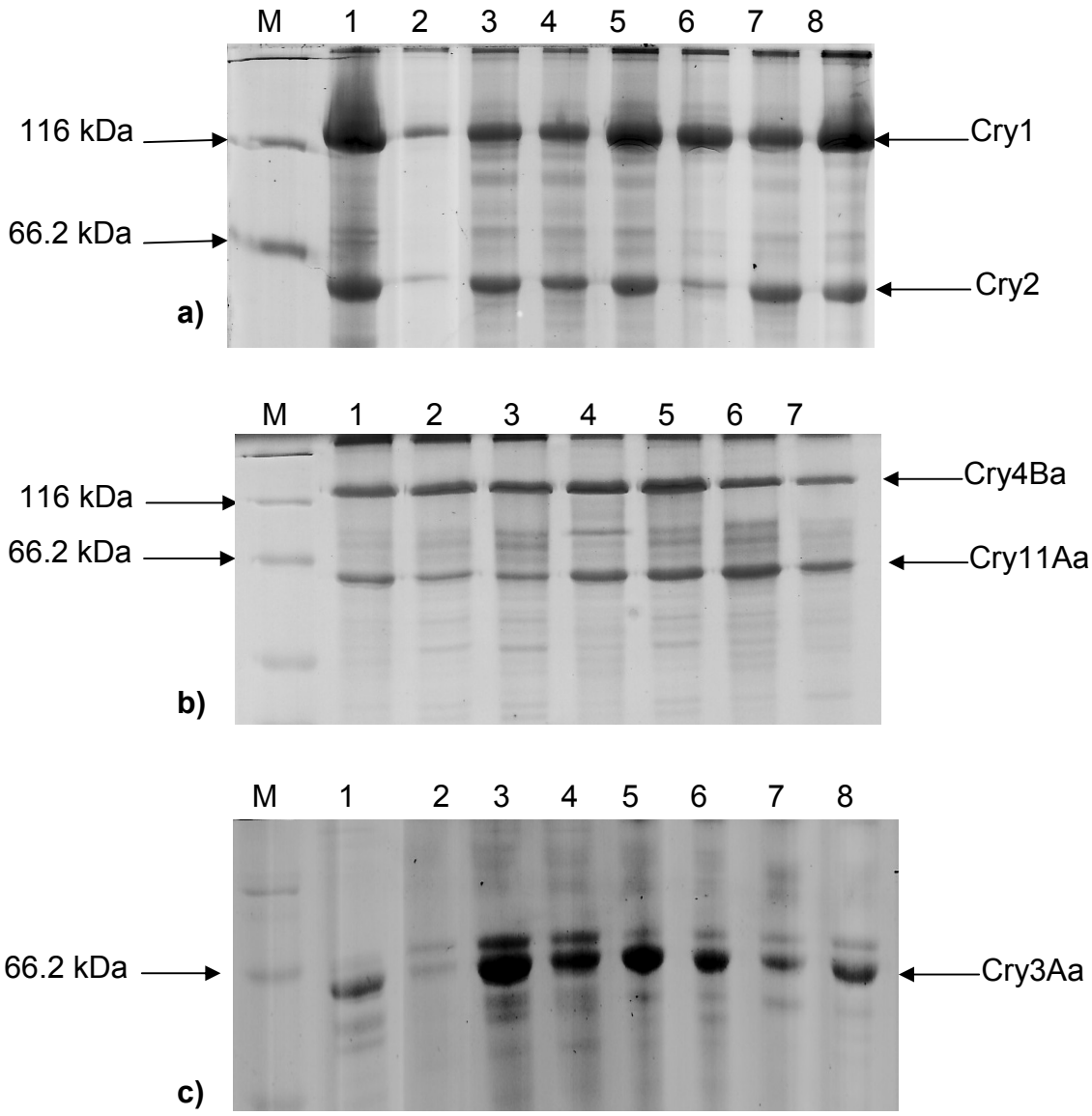


Figure 3. 13. Comassie stained SDS-PAGE showing the effect of C:N ratio on Cry1 and Cry2 biosynthesis by *Bt* 81(a), Cry4Ba and Cry11Aa biosynthesis by *Bt* HD500 (b) and Cry3Aa biosynthesis by *Bt* 3203 (c) after 24 h incubation. Unless otherwise indicated, the inocula were prepared in processed broiler litter. M: Molecular weight markers. Lane 1: DSM control (50 mM Pi); Lane 2: Unprocessed broiler litter; Lane 3: Processed broiler litter (C:N ratio 1.7); Lane 4: Processed broiler litter, DSM-grown inoculum; Lane 5: Processed broiler litter (C:N ratio 4); Lane 6: Processed broiler litter (C:N ratio 6), Lane 7: Processed broiler litter (C:N ratio 8), Lane 8: Processed broiler litter (C:N ratio 10).

In conclusion, the medium prepared from processed broiler litter was successfully used to cultivate all *Bt* stains and obtain bioinsecticidal proteins in high yields which were comparable or higher than to those that can be obtained on standard semi-synthetic media. These promising results stimulate further work such as the determination of viable cells as well as spores during fermentation along with the necessary insect bioassays which would prove the feasibility and utility of the new medium.

CHAPTER 4

CONCLUSION

- Among *Bt* strains employed in the present study (*Bt kurstaki* strain 81, *Bt israelensis* strain HD500 and *Bt tenebrionis* strain 3203, producing lepidoptera- and diptera-specific Cry1 and Cry2, diptera-specific Cry4Ba and Cry11Aa and coleoptera-specific Cry3Aa toxins, respectively), general suppression of toxin yields by high glucose concentration of 10 g/L indicated that carbon catabolite repression is a general phenomenon applying to the biosynthesis of different classes and types of crystal protein.

- Inorganic phosphate level was important for Cry4Ba, Cry11Aa and Cry3Aa biosynthesis while Cry1 and Cry2 production was not responsive to high Pi.

- Wastewater sludge-based media was convenient for the cultivation of *Bt* 81, but not for that of the other *Bt* strains tested.

- In this study, fruit residues were used for the first time as a raw substrate. Even when supplemented with Nutrient broth, a medium based on such residues was not suitable for *Bt* fermentations.

- Broiler litter seemed to be a much better substrate than fruit residues in that except for Cry4Ba, some degree of production of each toxin was observed at almost every stage of fermentation.

- Of different broiler litter pretreatments tested, solid/liquid separation followed by hydrolysis of solids in 2N HCl at 50^{o} C for 1 h (pretreatment IV) provided the most effective substrate processing as it resulted in the highest toxin yields.

- C:N ratio of unprocessed broiler litter was considerably lower than that of the processed broiler litter. Elemental analysis proved that pretreatment ensure a significant reduction in nitrogen content of the litter (by half).

- Experiments were conducted to optimize C:N ratio of processed broiler litter-based medium for each *Bt* strain. A C:N ratio of 4 was ideal for Cry1 and Cry2 production by *Bt* 81 as well as for Cry4Ba and Cry11Aa biosynthesis by *Bt* HD500 (Figure 3.13.a and b). For Cry 3Aa toxin biosynthesis, on the other hand, C:N ratio was found to be a very important parameter as C:N ratios higher than 4 decreased delta-endotoxin yield remarkably

- Processed broiler litter-based medium was successfully used to cultivate *Bt* stains and obtain bioinsecticidal proteins in high yields, even comparable to those that can be obtained in optimized standard media.

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APPENDIX A

COMPOSITION AND PREPARATION OF CULTURE MEDIA

DSM	**g/L**
Nutrient Broth	4
Glucose	5
K_2HPO_4	4.3545 (25 mM)
KH_2PO_4	3.4045 (25 mM)
$Ca(NO_3)_2$	0.118 (0.5 mM)
$MgSO_4$	0.0602 (0.5 mM)
$FeSO_4$	0.00278 (10 µM)
$MnCl_2$	0.00162 (10 µM)

Luria Broth	**g/L**
Luria Broth	25
Sterilized at 121 ^{0}C for 15 minutes.	

Nutrient Agar	**g/L**
Nutrient Broth	8
Agar	10
Sterilized at 121 ^{0}C for 15 minutes	

Luria Broth Agar	**g/L**
Luria Broth	25
Agar	15
Sterilized at 121 ^{0}C for 15 minutes	

APPENDIX B

SOLUTIONS AND BUFFERS

Acrylamide/Bis

Acrylamide	146 g
N.N'-Methylene-bis Acrylamide	4 g

Distilled water to 500 mL. Filtered and stored at 4 ^{0}C. Protected form light.

Tris HCl (1.5 M)

Tris base	54.45 g
dH_2O	150 mL

pH is adjusted to 8.8 with HCl, distilled water to 300 mL and stored at 4 ^{0}C.

Tris HCl (0.5 M)

Tris base	6 g
dH_2O	60 mL

pH is adjusted to 6.8 with HCl, distilled water to 100 mL and stored at 4 ^{0}C.

Tris-EDTA Buffer (TE) (Maniatis, 1989)

Tris	10 mM
EDTA	1 mM

pH is adjusted to 8.0 with HCl.

Sample Buffer

dH_2O	3 mL
Tris HCl (0.5 M)	1 mL
Glycerol	1.6 mL
SDS (10%)	0.4 mL
β- mercaptoethanol	0.4 mL
Bromophenol blue (0.5%, w/v) (in water)	0.4 mL

Running Buffer (5X)

Tris base	15 g
Glycine	72 g
SDS	5 g

Distilled water to 1 L. Stored at 4 ^{0}C.

Coomassie Blue R-250 Staining

Coomassie blue R-250	0.25 g
Methanol	125 mL
Glacial Acetic acid	25 mL
dH_2O	100 mL

Destaining Solution

Methanol	100 mL
Glacial Acetic acid	100 mL
dH_2O	800 mL

APPENDIX C

CHEMICALS AND THEIR SUPPLIERS

Chemicals

Acrylamide	Merck
Ammonium persulphate	AppliChem
Bovine Serum Albumin	Sigma
Bromophenol blue	Sigma
$CaCl_2.2H_2O$	Merck
$Ca(NO_3)_2.4\ H_2O$	Merck
Casamino acid	AppliChem
Coomassie Brillant Blue G-250	Merck
Coomassie Brillant Blue R-250	Sigma
EDTA	AppliChem
Ethanol	Botafarma
$FeSO_4.7H_2O$	Sigma
$Fe_2(SO_4)_3$	Sigma
Glacial Acetic acid	Merck
Glucose	Merck
Glycerol	Merck
Glycine	Merck
HCl	Merck
KH_2PO_4	Merck
K_2HPO_4	Merck

L-amino acids	Sigma
Luria Broth	Q-Biogene
Mercaptoethanol	Sigma
Methanol	Merck
$MgSO_4$	Sigma
$MgSO_4.7H_2O$	Merck
$MnSO_4.7H_2O$	Carlo Erba
$MnCl_2.2\ H_2O$	Merck
NaCl	Merck
NaOH	Merck
N, N'-Methylene bis-acrylamide	Sigma
Nutrient broth	Merck
Nutrient Agar	Merck
Peptone	Sigma
Sucrose	Merck
SDS	Merck
TEMED	Sigma
Tris Base	Merck
Tris-HCl	Merck
Yeast extract	Difco
$ZnSO_4.7H_2O$	Sigma

Enzymes

Lysozyme	MIB Fermentas

Size Markers

Protein molecular weight marker MIB Fermentas

β-galactosidase (116.2 kDa), bovine serum albumin (66.2kDa), ovalbumin (45 kDa), lactate dehydrogenase (35kDa), restriction endonuclease *Bsp*981 (25 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa) were used as the size markers.