Optimization of *Bacillus thuringiensis* subsp. *kurstaki* HD-73's Cry1Ac Toxin Production Parameters in Bioreactor Conditions and Assessment of HD-73's Biological Activity Against Tomato Leaf Miner (*Tuta absoluta*) (Meyrick) (Lepidoptera: Gelechiidae)

Gülin ÖZCAN

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Bacillus thuringiensis var. *kurstaki* HD-73 Irkının Cry1Ac Toksin Üretim Koşullarının Biyoreaktör Ortamında Optimizasyonu ve Domates Güvesine (*Tuta absoluta*) (Meyrick) (Lepidoptera: Gelechiidae) Karşı Biyolojik Etkinliğinin Değerlendirilmesi

Gülin ÖZCAN

Tez Danışmanı: Doç. Dr. Kıvanç BİLECEN

Biyoteknoloji Lisansüstü Programı

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By

Gülin ÖZCAN

Thesis Advisor: Assoc. Prof. Dr. Kıvanç BİLECEN

Graduate School of Biotechnology

Meram-KONYA

MAY, 2019

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis of the degree of Master

…….

Assoc. Prof. Dr. Kıvanç BİLECEN (Advisor)

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis of the degree of Master

…….

Prof. Dr. Cumhur ÇÖKMÜŞ

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis of the degree of Master

…….

Assist. Prof. Dr. Özgür GÜL

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis of the degree of Master

…….

Prof. Dr. Sencer BUZRUL

Director of the Institute of Graduate School of Biotechnology

Gülin ÖZCAN tarafından yüksek lisans tezi olarak sunulan " *Bacillus thuringiensis* var. *kurstaki* HD-73 Irkının Cry1Ac Toksin Üretim Koşullarının Biyoreaktör Ortamında Optimizasyonu ve Domates Güvesine (*Tuta absoluta*) (Meyrick) (Lepidoptera: Gelechiidae) Karşı Biyolojik Etkinliğinin Değerlendirilmesi" başlıklı bu çalışma KGTÜ Lisansüstü Eğitim ve Öğretim Yönetmeliği ile KGTÜ Fen Bilimleri Enstitüsü Eğitim ve Öğretim Yönergesi'nin ilgili hükümleri uyarınca tarafımızdan değerlendirilerek savunmaya değer bulunmuş ve ../../2019 tarihinde yapılan tez savunma sınavında aday oybirliği/oyçokluğu ile başarılı bulunmuştur.

Jüri Üyeleri:

İmza:

Jüri Başkanı : Doç Dr. Kıvanç BİLECEN

Raportör Üye : Dr. Öğr. Üyesi Özgür GÜL

Üye : Prof. Dr. Cumhur ÇÖKMÜŞ

This study titled “Optimization of *Bacillus thuringiensis* subsp. *kurstaki* HD-73’s Cry1Ac Toxin Production Parameters in Bioreactor Conditions and Assessment of HD-73’s Biological Activity Against Tomato Leaf Miner (*Tuta absoluta*) (Meyrick) (Lepidoptera: Gelechiidae) ” and presented as Master Thesis by Gulin OZCAN has been evaluated in compliance with the relevant provisions of KFAU Graduate Education and Training Regulation and KFAU Institute of Science Education and Training Direction and jury members written below have decided for the defense of this thesis and it has been declared by consensus/majority of votes that the candidate has succeeded in thesis defense examination dated.

Jury Members:

Signature:

Head : Assoc. Prof. Dr. Kıvanç BİLECEN

Rapporteur Member : Asst. Prof. Dr. Özgür GÜL

Member : Prof. Dr. Cumhur ÇÖKMÜŞ

ÖZET

Bacillus thuringiensis var. *kurstaki* HD-73 Irkının Cry1Ac Toksin Üretim Koşullarının Biyoreaktör Ortamında Optimizasyonu ve Domates Güvesine (*Tuta absoluta*) (Meyrick) (Lepidoptera: Gelechiidae) Karşı Biyolojik Etkinliğinin Değerlendirilmesi

ÖZCAN, Gülin

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Tez Danışmanı: Doç. Dr. Kıvanç BİLECEN

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Domates güvesi *Tuta absoluta,* Türkiye ve dünya genelinde domates için tarla ve sera koşullarında en tahrip edici bitki zararlılarından biridir. Bu zararlının kontrolü esas olarak kimyasal insektisitler kullanılarak sağlanmaktadır. Fakat, bu kimyasalların kullanımı, çevre kirliliği ve insan sağlığı etkileri dahil olmak üzere çeşitli sorunlara neden olmuştur. Ayrıca diğer birçok zararlı böcek gibi *T. absoluta* da son yıllarda kimyasal insektisitlere karşı direnç geliştirmiştir. Kimyasal insektisitlere alternatif olarak, *Bacillus thuringiensis* suşları ve entomopatojenik Cry toksinleri, uzun yıllardır çeşitli böcek türlerine karşı biyolojik kontrol ajanı olarak kullanılmaktadır.

Bu tez çalışmasında, *B. thuringiensis kurstaki* HD-73 suşu, hem konvansiyonel kültürde hem de biyoreaktör ortamında *T. absoluta'ya* karşı biyolojik kontrol ajanı olarak kullanılmak üzere optimize edilmiştir. Bu amaçla çeşitli karbon, azot ve mineral tuz kaynakları denenmiştir. Yapılan denemeler sonucunda NYSM besiyerinin, toksin üretimi açısından en etkili kültür ortamı olduğu bulunmuştur. Ayrıca, biyolojik aktivite testleri, *T. absoluta* larvaları üzerinde Cry1Ac toksinleri içeren HD-73 sporları kullanılarak gerçekleştirilmiştir. Bu biyolojik deneylerde, *B. thuringiensis* HD-73 sporlarını içeren taze kültürler ile LC_{50} değeri hesaplanmış ve denemelerde %100 ölüm oranı yakalanabilmiştir.

Anahtar sözcükler: *Bacillus thuringiensis* subsp. *kurstaki* HD-73, *Tuta absoluta*, domates güvesi, biyolojik kontrol

ABSTRACT

Optimization of *Bacillus thuringiensis* subsp. *kurstaki* HD-73's Cry1Ac Toxin Production Parameters in Bioreactor Conditions and Assessment of HD-73's Biological Activity Against Tomato Leaf Miner (*Tuta absoluta*) (Meyrick) (Lepidoptera: Gelechiidae)

OZCAN, Gulin

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Supervisor: Assoc. Prof. Dr. Kivanc BILECEN

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Tomato leaf miner, *Tuta absoluta*, is one of the most harmful pests for tomato both in farm and greenhouse conditions in Turkey and many other countries. Control of this pest mainly achieved by using chemical insecticides, which can cause environmental pollution and have human health effects due to its residues on final products. Besides, like many other pests, *T. absoluta* has developed resistance to chemical insecticides over the past years. As an alternative to chemical insecticides, *Bacillus thuringiensis* strains and their entomopathogenic Cry toxins have been used as a biological control agent for many years now against various insects.

In this thesis work the growth and toxin production conditions of *B. thuringiensis* subsp. *kurstaki* HD-73 have been optimized both in conventional culture and bioreactor settings to be used as a biological control agent against *T. absoluta*. To this end various carbon, nitrogen and mineral salt sources were tried. Nutrient Yeast Extract Salt Medium (NYSM) was found to be the most effective for toxin production under our conditions tested. Biological activity tests were also performed on *T. absoluta* larvae using HD-73 spores containing Cry1Ac toxins. In these biological assays LC_{50} value was calculated and we have reached a 100% death rate with fresh preparations of *B. thuringiensis* subsp. *kurstaki* HD-73 spores containing Cry toxins.

Keywords: *Bacillus thuringiensis* subsp. *kurstaki* HD-73, *Tuta absoluta*, tomato leaf miner, biological control

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Lastly, this thesis is dedicated to my precious family; to my angels and my deceased father who is the only man i love… Everything is for you and thanks to you. Love you so much…

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Dedicated to my family with all my heart…

YEMİN METNİ

Yüksek lisans tezi olarak sunduğum "*Bacillus thuringiensis* var. *kurstaki* HD-73 Irkının Cry1Ac Toksin Üretim Koşullarının Biyoreaktör Ortamında Optimizasyonu ve Domates Güvesine (*Tuta absoluta*) (Meyrick) (Lepidoptera: Gelechiidae) Karşı Biyolojik Etkinliğinin Değerlendirilmesi " adlı çalışmanın tarafımdan bilimsel ahlak ve geleneklere aykırı düşecek bir yardıma başvurmaksızın yazıldığını ve yararlandığım eserlerin bibliyografyada gösterilenlerden oluştuğunu, bunlara atıf yapılarak yararlanılmış olduğunu belirtir ve bunu onurumla doğrularım.

20/05/2019

Gülin ÖZCAN

TEXT OF OATH

I declared and honestly confirm that my study titled “Optimization of *Bacillus thuringiensis* subsp. *kurstaki* HD-73’s Cry1Ac Toxin Production Parameters in Bioreactor Conditions and Assessment of HD-73’s Biological Activity Against Tomato Leaf Miner (*Tuta absoluta*) (Meyrick) (Lepidoptera: Gelechiidae)” and presented as Master’s Thesis has been written without applying to any assistance inconsistent with scientific ethics and traditions and all sources I have benefited from are listed in bibliography and I have benefited from these sources by means of making references.

20/05/2019

Gülin ÖZCAN

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1. INTRODUCTION

Turkey is one of the largest tomato producers in the world with 12,6 million tons and 239.880 US dollars per year (FAO, 2016). Starting from 2011 *Tuta absoluta*, commonly known as tomato leaf miner, has become one of the most harmful tomato pests in Turkey and can cause product losses 80-100% in dense populations (bmae.gov.tr). Chemical insecticides are commonly used to deal with *T. absoluta*; however, they pose significant risks to both human and environment. Also, as they precipitate on products, tomatoes with chemical precipitation are often rejected by importers causing economic loss. Biological control strategies, with their advantages, have become quite popular as an environmentally friendlier option in recent years.

Bacillus thuringiensis – a Gram-positive endospore forming bacterium, strains have long been commercially used as biological control agents against various insects including the ones causing health problems in humans like mosquitos and the ones causing plant damages (Höfte & Whiteley, 1989). *B. thuringiensis*' entomopathogenic effects comes from the parasporal crystals that are produced during sporulation. Cry toxins, one of two such toxin proteins, are dissolved in the middle intestine regions of sensitive insects. At this stage it is still inactive and called Cry prototoxin. The cry prototoxin is then activated by the proteases which have been synthesized by the bacteria or are present in the insect gut. The aminopeptidases found in insect cell surfaces, cadherin proteins, alkaline phosphatase enzymes and ABC transporter proteins have been identified as receptor proteins of Cry toxin (Bravo, Likitvivatanavong, Gill, & Soberón, 2011). These proteins also define the host range and specificity of the Cry toxin host. For example, the *B. thuringiensis* HD73 strain has a specific effect on moth butterflies by producing Cry1Ac (Navon, 2000). When the larvae are fed with these toxins, it results with larval death (Adang, Crickmore, & Jurat-Fuentes, 2014; Xue et al., 2008).

In this thesis work we optimized the growth and toxin production parameters of *B. thuringiensis* subsp. *kurstaki* HD-73 (Btk-HD73) both in conventional culture and bioreactor settings. To this end we have tried various carbon (molasse, whey), nitrogen and mineral salt sources (TBL, NSYM with Mn_2, Mg_2) as well as pH and aeration conditions. Nutrient Yeast Extract Salt Medium (NYSM) was found to be the most effective for toxin production under our conditions tested. Biological activity tests were

also performed on *T. absoluta* larvae using Btk-HD73 spores containing Cry1Ac toxins. In these biological assays we have reached an LC_{50} value of 100% with fresh preparations.

2. BACKGROUND INFORMATION

2.1. Plant Protection

In food production, pest damage is the most important threat in terms of the protection of the crops, fruits and vegetables. Insects and fungi have led to economic losses in agricultural production over the years (Pérez-García, Romero, & de Vicente, 2011).

Plant protection from insect species is predominantly achieved by using chemical insecticides until today. The use of chemical insecticides has caused some of environmental, human health and ecological problems which means they have an negative effect on nontarget organisms. Human health problems such as cancer and immune system disorders are serious diseases, which are very common (Brucker-Davis, 1998; Gilden, Huffling, & Sattler, 2010). A number of scientific studies showed that these chemicals cause endocrine disruption (Brucker-Davis, 1998; Gilden et al., 2010), neurotoxicity (Alavanja, Hoppin, & Kamel, 2004) and breast cancer in particular (Payne, Scholze, & Kortenkamp, 2001). A recent World Health Organization and United Nations Environmental Program stated that, there are 1 million human pesticide poisonings each year in the world (WHO/UNEP 1989),(Pimentel et al., 2019). Environmental contamination and ecological imbalance are the long term effects of insecticides, as well (Igbedioh, 1991). Each year, thousands of animals are poisoned by pesticides; their meat, milk, and egg products are also contaminated (Pimentel et al., 2019). In addition, beneficial natural enemies are affected by pesticides (Croft,1990). According to the one study, 20% of the honeybee colonies were lost due to the pesticide exposure (Pimentel et al., 2019). Because of the all these reasons, there is a growing demand for new and safer methods for control strategies (Pérez-García et al., 2011).

In recent years, biological control methods have emerged as an alternative to chemical pesticides. Use of natural antagonists against pests or plant diseases gained popularity as an environmentally friendly approach (Pérez-García et al., 2011). There are numerous studies and numerous commercial products involving different microorganisms as biological control agents. There are many different microbial pesticides produced with *Bacillus thuringiensis* against lepidoptera, *B. thuringiensis* subsp. *israelensis* against mosquito, entomopathogenic fungi against broad range of

insects and infections, baculoviruses and protozoa (Starnes, Liu, & Marrone, 2014) as it shown in the Table 1. One of the main advantages of microbial insecticides is their specificity. Each microorganism show a limited activity spectrum and can kill only certain insect species (Betz, Hammond, & Fuchs, 2000; Bravo et al., 2011).

Table 1 The list of microbial pesticides.

Microorganism	Yr registered	Target Pest
Bacteria		
Bacillus popilliae/lentimorbus Dutky	1948	Japanese beetle larvae
B. thuringiensis	1961	Lepidoptera larvae
Agrobacterium radiobacter (Beijerinck and van Delden) Conn	1979	Crown gall disease
B. thuringiensis israelensis	1981	Mosquito/blackfly larvae
B. thuringiensis aizawai	1981	Lepidoptera larvae
Pseudomonas fluorescens (Trevisan) Migula	1988	*Pythium/Rhizoctonia*
B. thuringiensis tenebrionis	1988	Certain beetle larva
B. thuringiensis EG 2348	1989	Gypsy moth larvae
B. thuringiensis EG 2371	1989	Lepidoptera larvae
B. thuringiensis EG 2424	1990	Lepidoptera/Coleoptera larvae
Killed, engineered Pseudomonas with *B. thuringiensis*	1991	Lepidoptera/Coleoptera larvae
Viruses		
Heliothis NPV	1975	*Heliothis* complex
Tussock moth NPV	1976	Douglas-fir tussock moth larvae
Gypsy moth NPV	1978	Gypsy moth larvae
Pine sawfly larvae	1983	Pine sawfly larva
Fungi		
Hirsutella thompsonii Fisher	1981	Citrus rust mite
Phytophthora palmivora Butler	1981	Citrus strangler vine
Colletotrichum gloeosporoides Fenzig	1982	Northern joint vetch
Trichoderma harzianum Rifai/ *Trichoderma polysporum* (Link) Rifai	1989	Wood rot
Protozoa		
Nosema locustae Canning	1980	Grasshoppers

B. thrungiensis is probably the most used microorganism as insectide and present a number of advantages through its cry toxin which is a parasporal crystals producing during sporulation simultaneously (Bravo et al., 2011; Heimpel, 2003). While *B. thrungiensis* microorganisms or its spores can directly be used in formulations, toxin genes can be inserted into various plant species to give resistance (Heimpel, 2003).

Cry toxin proteins have no known adverse effect on organisms other than their intended targets due to their narrow activity spectrum and very target-specific nature. Since 1961, many Bt and Cry toxin containing product have been registered both in the United States and in Europe; and so far no human health related or any environmental

problems have been reported (Betz et al., 2000)(EPA 98, IPCS 2000). Studies to assess Cry (Cry1, Cry2 and Cry3) protein's potential health effect showed no adverse outcome on mammals even with high oral doses. In addition, it has been shown that these proteins are degraded in gastric fluid, and also, through immunocytochemical analyses it has been shown that Cry1A toxin family don't have any binding sites in mammalian systems (Betz et al., 2000; J.T., C.R., & Sjoblad, 1995).

2.2. *Bacillus thuringiensis*

B. thuringiensis is a member of the genus Bacillus, gram-positive, spore-forming soil bacterium with entomopathogenic properties (Höfte & Whiteley, 1989; Tabashnik, 1994). The organism was first isolated in 1901 by the Ishiwata Shigetane from a silkworm (*Bombyx mori*). Then, a decade later Ernst Berliner, isolated a bacterium from a diseased flour moth larva in German state Thuringia (*Anagasta (Ephestia) kuehniela).* Thus, the organism named as "*Bacillus thuringiensis*" (H. T. Dulmage, Boening, Rehnborg, & Hansen, 1971; Höfte & Whiteley, 1989; Schnepf, Crickmore, Rie, et al., 1998). Crystal inclusion bodies were also noticed in *B. thrungiensis* spores. First insecticidal trials were performed against the European corn borer (*Ostrinia nubilalis*). But later it was noticed that not the spores but parasporal crystals were directly related with insecticidal activity (Osman et al., 2015).

Entomopathogenic properties of *B. thrungiensis* depends on insecticidal parasporal inclusion which is synthesized during sporulation (Adang et al., 2014; Bravo, Gill, & Soberón, 2007; Debro, Fitz-James, & Aronson, 1986; Höfte & Whiteley, 1989; Somerville & James, 1970).

Inclusions show variations in their numbers, shape and compositions. In general, there is one inclusion per cell, but sometimes two or more inclusions can also be seen. Inclusions' shapes are bipyramidal; though rarely, irregular shapes can also be seen (I. Aronson, Beckman, & Dunn, 1986; Debro et al., 1986). These inclusions consist of proteins which are called Crystal (Cry) or Cytolitic (Cyt) toxins. Crystal proteins are inside the sporangium (is an enclosure in which spores are formed) and is being stored outside the endospore as shown in Figure 1 (Bechtel & Bulla, 1976).

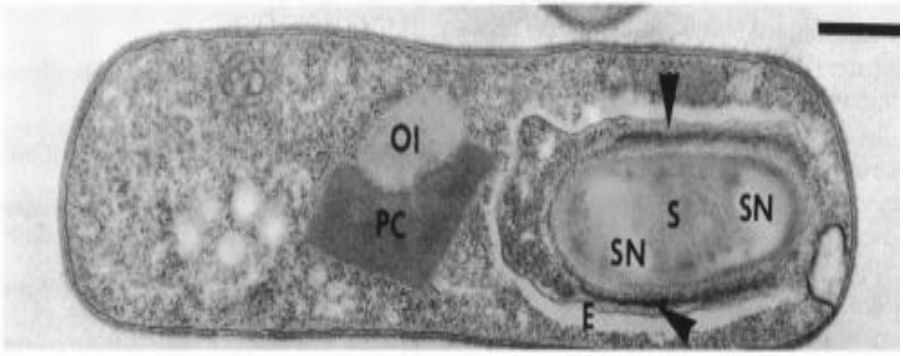


Figure 1 Parasporal crystal formation of *Bacillus thuringiensis.*
Parasporal crystal (PC) and forespore (F) at one end and the ovoid inclusion (OI) lateral sides of spore (S) exosporium (E) spore nucleoid (SN) (Bechtel & Bulla, 1976).

For insecticide activities we can talked about three main types of *B. thuringiensis* toxins: α-exotoxin (a heat labile exotoxin), β-exotoxin (a heat stable exotoxin) and delta δ-endotoxin (also known as crystal protein). α and β exotoxins act on a wide variety of insect orders and they do not have any specificity. Crystal proteins (delta endotoxins) can also act on various orders like Lepidoptera, Coleoptera and Diptera that cause agricultural and health related problems, but each different cry toxin has a narrow host range and kill only certain (Bhowmik, 2012; Höfte & Whiteley, 1989). Cry protein's toxin activity depends on its chemical structure and show variations within different *B. thuringiensis* strains. Besides conventional serological and biochemical methods used to classify *B. thuringiensis* strains, H serotyping and immunological reaction to the flagellar antigens are also used for classification. Until today, 69 H serotypes and 82 serovars of *B. thuringiensis* have been categorized (Balasubramanian et al., 2002). Each *B. thuringiensis* strain has lethal effect on different lepidopteran species (Höfte & Whiteley, 1989). These characteristics have made *B. thuringiensis* as a successful bioinsecticide agent for more than 40 years (Bhowmik, 2012; Schnepf, Crickmore, Rie, et al., 1998). For example, few *B. thuringiensis* subspecies known as *israelensis* are toxic to mosquitoes (Cokmus & Elçin, 1995; Thomas & Ellar, 1983) while some other isolates active against nematodes and protozoa (Schnepf, Crickmore, Van Rie, et al., 1998).

At the beginning commercially used *B. thuringiensis* products had mostly been derived from the subspecies *kurstaki* strain HD-1 like DIPEL and BIOBIT (H. T. Dulmage et al., 1971; Schnepf, Crickmore, Van Rie, et al., 1998). Then, through the

commercial improvement studies different strains have been developed to overcome issues like resistance and insufficient toxin activity.

2.2.1. Ecology and Prevalence

B. thuringiensis strains have been isolated from soil, insects (Kaelin, Morel, & Gadani, 1994; Schnepf, Crickmore, Rie, et al., 1998), coniferous leaves (Mizuki et al., 1999; Smith & Couche, 1991) and aquatic environments (Iriarte, Porcar, Lecadet, & Caballero, 2000) worldwide through the heat application for spores or antibiotic selection(DeLucca II, Simonson, & Larson, 2010; Schnepf, Crickmore, Rie, et al., 1998).

The organism is found in soil as a saprophyte, spores persist in soil also and vegetative growth take in place when nutrients are present (Osman et al., 2015). *B. thuringiensis* spores can be found in nature even after several years of application (Schnepf, Crickmore, Rie, et al., 1998).

2.2.2. Sporulation

B thuringiensis life cycle includes two phases; vegetative cell growth and sporulation. Sporulation is developed by seven stages and includes asymmetric cell division (Bechtel & Bulla, 1976). Stage I includes axial filament formation which there is no connection of mesosomes with the nucleoid. In stage II, forespore septum formation occurs involving mesosomes and in stage III, inclusion and parasporal crystal become apparent and formation of forespore takes in place. Stages IV to VI includes formation of exosporium, cell wall, cortex, and spore coats. And finally in stage VII, spore maturation occurs (Bechtel & Bulla, 1976).

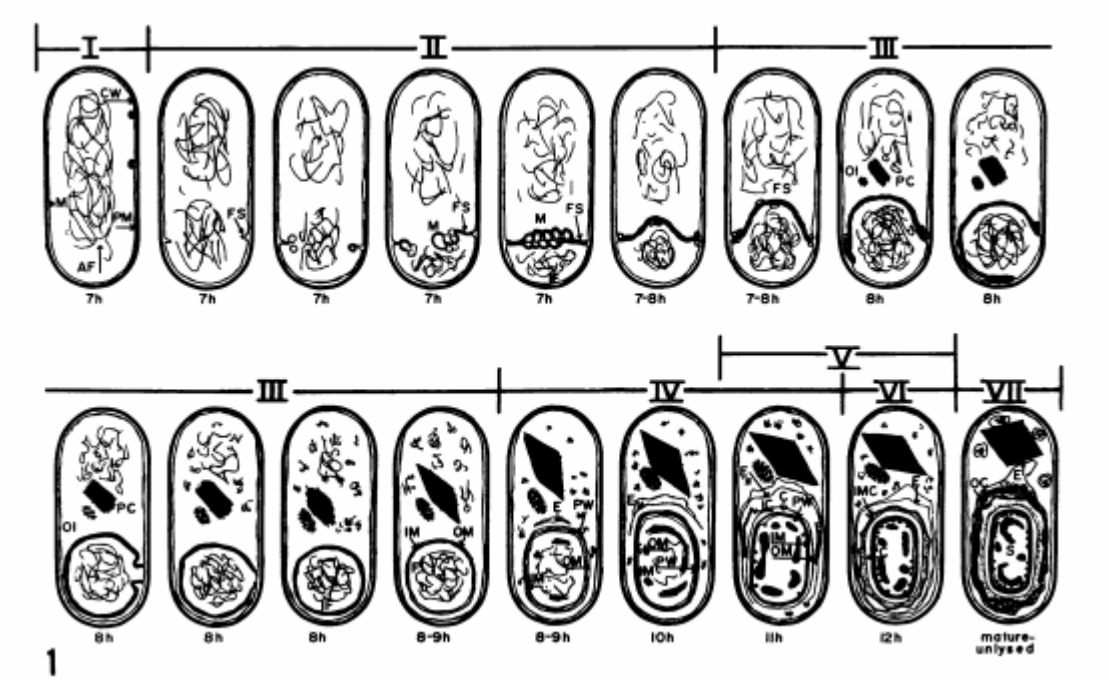


Figure 2 Diagramatic scheme of sporulation in *Bacillus thuringiensis*.
Abbreviations: M, mesosome; CW, cell wall; PM, plasma membrane; AF, axial filament; FS, forespore septum; IF, incipient forespore; OI, ovoid inclusion; PC, parasporal crystal; F, forespore; IM, inner membrane; OM, outer membrane; PW, primordial cell wall; E, exosporium; LC, lamellar spore coat; OC, outer spore coat; C, cortex; IMC, incorporated mother cell cytoplasm; S, mature spore in an unlysed sporangium (Bulla,1976).

2.2.3. *Bacillus thuringiensis* Genome

B. thuringiensis have a genome of 2.4 to 5.7 million bp (Carlson & Kolsto, 1993; Schnepf, Crickmore, Rie, et al., 1998). Most of the strains contain several plasmids with sizes ranging from 2 kb to 200 kb. Same plasmids can be found among different subspecies and strains. It is also important to note that although parasporal crystals are generally encoded by plasmids, these sequences can also be found in *B. thrungiensis* choromosomes (Carlson & Kolsto, 1993; Schnepf, Crickmore, Rie, et al., 1998). *B. thuringiensis* species have a large variety of transposable elements both insertion sequences and transposons. Several insertion sequences have been found in *B. thuringiensis* strains and many of these carry protoxin genes (Debro et al., 1986).

2.2.4. The cry genes

Until today, over 100 *cry* gene sequences have been classified into 50 subgroups according to their sequence (Bravo et al., 2007, 1998; Crickmore et al., 1998). In *B.*

thuringiensis, cry genes are expressed in stationary phase. Then synthesized insecticidal proteins are stored and accumulated in the mother cell as a crystal inclusion, which can make up to 20 to 30% of the dry weight of a sporulated cell (Agaisse & Lereclus, 1995). Crystal protein synthesis is controlled by different mechanisms at transcriptional, posttranscriptional and posttranslational levels (Schnepf, Crickmore, Rie, et al., 1998).

2.2.4.1. Transcriptional Mechanisms

The *cry* genes have been known as sporulation-specific genes. However, expression of the c*ry3Aa* gene have showed that this is not accurate in all cases. In other words, stationary phase genes expression can be dependent on sporulation or not (Agaisse & Lereclus, 1995).

2.2.4.1.1. Sporulation-dependent *cry* gene expression

In sporangium, endospore development process is controlled at the transcriptional level by the activation of six different sigma factors that bind to RNA polymerase to specify target. The primary sigma factor of vegetative cells is σ^A and five other factors (σ^H, $\sigma^{F,}$ σ^E, σ^G and σ^K) are stimulated during development. The σ^A and σ^H factors are active in predivisional cell, σ^E and σ^K are active in the mother cell and σ^F and σ^G are active in the forespore (Agaisse & Lereclus, 1995; Höfte & Whiteley, 1989; Sanchis et al., 1999).

In *cry1A* gene two transcription sites have been mapped as Bt1 and Bt2, which are overlapping promoters. σ^{35} and σ^{28} are responsible direct transcription of Cry1Aa from Bt1 and Bt2, respectively. Besides c*ry1A* gene, Cry1B, Cry2A and CytA also contain Bt1 and Bt2. Consensus sequences recognized by RNA polymerase containing σ^E and σ^K sigma factors have been deduced from promoter regions of these genes. *Cry4A*, *cry4B* and c*ry4D* are also thought to be sporulation specific genes (Agaisse & Lereclus, 1995; Schnepf, Crickmore, Rie, et al., 1998).

2.2.4.1.2. Non-sporulation dependent *cry* gene expression

The *cry3A* gene is a characteristic example of non-sporulation dependent expression mechanism which is active against coleopteran species. The *cry3A* promoter is expressed throughout the vegetative stage and activated at the end of the exponential growth

(Agaisse & Lereclus, 1995; Schnepf, Crickmore, Rie, et al., 1998). Different from Bt1 and Bt2, promoter of *cry3A* gene resembles to the primary sigma factor of vegetative cells known as σ^A. Unlike other Cry toxin proteins, c*ry3A* expression is not dependent on sporulation specific sigma factors (Schnepf, Crickmore, Rie, et al., 1998).

2.2.5. *Bacillus thuringiensis* Toxins Used in the Plant Protection Studies

Parasporal crystals are classified into two groups as crystal (Cry) and Cytolytic (Cyt) toxins (Adang et al., 2014; Höfte & Whiteley, 1989). The Cyt toxins are composed of a single a-b domain (Bravo et al., 2007). These proteins are capable of lysing a wide range of cell types in vitro and show specific activity on Diptera family (Adang et al., 2014; Bravo et al., 2011)

Cry proteins are also known as δ-endotoxins and they consist of nearly 300 different members categorized according to their primary amino acid sequences (Adang et al., 2014; Bravo et al., 2007). Some members, although they show very low amino acid sequence similarity, they may still have similar mechanism due to their three dimensional structure (Bravo et al., 2011).

The largest Cry protein family is the 3D-Cry, with at least 40 different groups and more than 200 different gene sequences. The three-dimensional structure of seven different 3D-Cry toxins have been solved, Cry1Aa, Cry1Ac, Cry2Aa, Cry3Aa, Cry3Ba, Cry4Aa, Cry4Ba (Pigott & Ellar, 2007).

Cry δ-endotoxins consist of three domains (Figure 3). Domain-1 contains a bundle of seven antiparallel alpha-helices (Bravo et al., 2007; Pigott & Ellar, 2007). The central helix is hydrophobic and outer helices are amphipathic. Most of the helices are capable of spanning a hydrophobic membrane. It is important to note that, the domain-1 is indicated in membrane insertion and pore formation due to the these properties (Bravo et al., 2011). Domain-2 consists three anti-parallel beta-sheets and form a beta prism. The lengths of these beta strands have been shown to vary between Cry families and this presumably indicates the Cry toxins' specificity (Pigott & Ellar, 2007).

Domain-3 is a beta sandwich (Bravo et al., 2013, 2011). Of two anti-parallel β-sheets these are packed together with a jelly roll topology (Pigott & Ellar, 2007). Together with domain-2, domain-3 is also implicated in insect specificity through the receptor binding (Bravo et al., 2011).

An analysis for the phylogenetic relations of these three domains indicated that Cry toxin variability had arisen by two processes: evolution of the three functional domains and by domain-3 swapping among toxins (Bravo et al., 2007, 2013, 2011). Domain-2 and -3 have been shown to confer specificity to Cry toxins for binding to insect midgut proteins as we know. Domains-1 and -2 have evolved together. However, the analysis of domain-3 sequences, indicated different topology. The independent evolution of the three structural domains and domain-3 swapping caused selection of proteins with similar activation process but different specifities among insect species (Bravo et al., 2007). In fact, in vitro construction of hybrid Cry proteins through altering domain-3, such as Cry1Ab hybrid toxin that contains the domain-3 of Cry1C toxin (1Ab-1Ab-1C) was demonstrated that (De Maagd, Bravo, & Crickmore, 2001) this hybrid toxin exhibited much more insecticidal activity against *Spodoptera exigua* larvae than other proteins (Bravo et al., 2007).

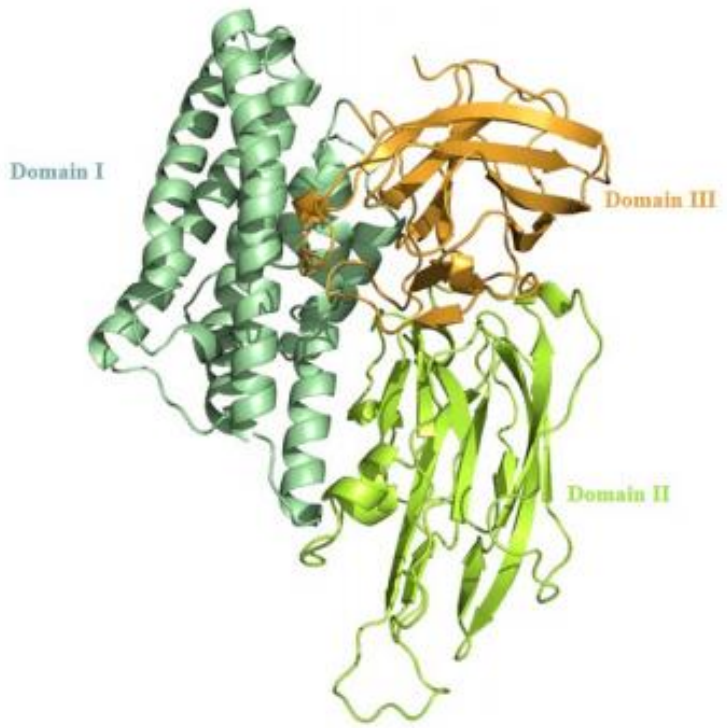


Figure 3 Crystal structure of Cry1A toxin protein.
Domain 1, domain 2, and domain 3 are shown in red, green, and blue, respectively. The N-terminal protoxin domain of Cry2Aa is shown in yellow (Xu,2014).

2.2.6. Mechanism of Action

The crystal protein Cry1Ac, is a 133.3 kDa solubilized polypeptide and a protoxin. This protoxin is converted into 60 kDa active toxins in the larval midgut (Bietlot et al., 2015). Cry1Ac toxins are produced during sporulation and they show selective specificity and activity on different insect species (Bietlot et al., 2015).

Insect specificity depends on the specific receptor binding of Cry toxins to surface proteins, which are in the microvilli membrane of larvae midgut cells (I. Aronson et al., 1986; Pigott & Ellar, 2007). These binding sites are cadherin and cadherin-like proteins such as GPI-anchored alkaline phosphatase (ALP) and glycosylphophatidyl-inositol (GPI)-anchored aminopeptidase-N (APN) (Bravo et al., 2007, 2011). For example, a type of Cry1A toxin binds to cadherin proteins of *Helicoverpa armigera* species in their midgut. In the case of APN, proteins related to different subfamilies of lepidoptera including *Helicoverpa armigera. Heliothis virescens, Lymantria dispar, Manduca sexta* and *Plutella xylostella* have been found to bind Cry1 toxins (Pigott & Ellar, 2007).

As previously mentioned, Cry1A proteins are produced as crystal inclusion protoxins and in the midgut they become active after being cleaved from their interchain disulfide bonds. These solubilized protoxins are processed through endogenous *B. thuringiensis* proteases in larvae gut fluids to an active toxin core resulting resistant toxin proteins against further proteolysis (Adang et al., 2014).

The activated toxin binds to insect gut Cry-binding proteins (Bravo et al., 2011). Toxins from different *B. thuringiensis* strains bind different Cry-binding proteins in susceptible insects. In the case of Cry1Ac toxin, it binds to APN receptor through the domain-3, which recognizes N-acetylgalactosamine (Ga1HNAc). In contrast Cry1Aa and Cry1Ab toxins show no Ga1NAc binding specificity (Verplaetse, Slamti, Gohar, & Lereclus, 2017). As Cry toxins target enterocytes, gut epithelial barrier becomes weaker and then toxin can access to haemocoel. Toxins generate pores in the cell membrane and osmotic balance is destabilized resulting cellular swelling and cell lysis at the end (Höfte & Whiteley, 1989). This leading to membrane insertion, pore formation and finally insect death (Xue et al., 2008). During this period, gut physiological conditions change, and this change enables *B. thuringiensis* spores to germinate in the haemolymph of larvae (Adang et al., 2014).

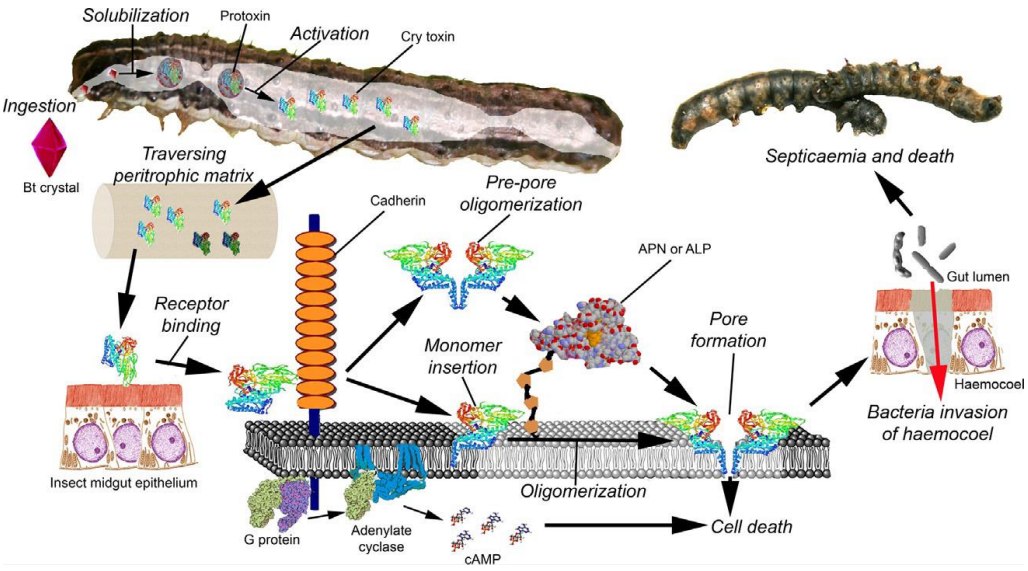


Figure 4 Representation of the current models of Cry toxin action in the insect midgut. (Adang,2014).

2.2.7. Application of Cry Proteins for Plant Protection

2.2.7.1. *Bacillus thuringiensis* as a biopesticide

B. thuringiensis is being used as a biological pest control agent for a long time (Bravo et al., 2011; Sanahuja, Banakar, Twyman, Capell, & Christou, 2011) In the beginning, *B. thuringiensis* products did not show sufficient effect in comparison with chemical pesticides. For this reason product development studies concentrated on increase of toxicity through the strain improvement (Sanahuja et al., 2011). Also, formulation studies became important as for a successful product, suspension agents, preservatives, UV screening agents should be added. Moreover, a successful application of *B. thuringiensis* depends on proper timing of larval stage and weather conditions (Bauce, Carisey, Dupont, & van Frankenhuyzen, 2009).

Although Cry proteins have 70 groups and a lot of subgroups, only a small number of these proteins shows toxicity against insect species and used for bioinsecticide products such as HD-1 and HD-73 strains (Reinoso-Pozo, Del Rincón-Castro, & Ibarra, 2016). *B. thuringiensis* products obtained from a few wild-type strains such as *B. thuringiensis* subsp. *kurstaki* HD1 and HD73. HD1 strain expresses Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa proteins; whereas HD73 expresses Cry1Ac. *B. thuringiensis* subsp. *aizawai* HD137, on the other hand, produces a different set of Cry toxins such as Cry1Aa, Cry1B,a Cry1Ca and Cry1Da. Due to this wide range, *B. thuringiensis* products are effective for controlling many different leaf-feeding lepidopterans (Navon, 2000) and to obtain an extensive impact, commercial products contain a family of Cry1 and Cry2 type toxins.

In Turkey, there is a number of licensed commercial *B. thuringiensis* containing products such as RAPAX, DIPEL DF, FLORBAC WG, BIOBIT WP, BIO-T PLUS, REBOUND WP and AGREE WG (bku.tarim.gov.tr). These products have different *B. thuringiensis* contents and therefore are used for different targets. For example, DIPEL contains HD-1 strain, which is not very effective against *T. absoluta*. HD73 strain produces a 133.3 kDa Cry1Ac insecticidal crystal protein only and this shows better activity against many insect species including *T. absoluta* (H. T. Dulmage et al., 1971; T. Dulmage, 1971; Sabbour & Soliman, 2014) compared to HD-1. Besides *T. absoluta*, HD-73 strain also shows efficient activity on *Manduca sexta, Heliothis virescens, Helicoverpa*

armigera, Spodopreta littoralis and *Spodoptera exigua* (A. I. Aronson, Geng, & Wu, 1999; Foda, 1981).

These products, however, are all imported to Turkey and to our knowledge currently there isn't any local producer exist.

2.2.8. Production of *Bacillus thuringiensis*

To obtain a successful product, content of the fermentation medium is crucial. Because of the expensiveness of *B. thuringiensis* products, the cost of materials and sporulation and growth condition of *Bacillus* depend on nutrition state and reflect its control mechanism, a variety of alternative inexpensive raw materials become important in fermentation cultures for cost-effective *B. thuringiensis* biopesticides production. Researchers have interested in increasing spore counts and toxin stabilization studies (Kaur, 2000). Carbon, nitrogen sources, and minerals has been used for medium. Carbon sources include; corn products, starch, dextrose, and nitrogen sources include corn step liquor, soybean, yeast, casein (Couch & Ross, 1980).

The growth of *B. thuringiensis* take place in the pH range of 5.5–8.5 (Içgen, & Özcengiz, 2002) and optimum temperature for growth and toxin production is 30°C (Özkan, Dilek, Yetis, & Özcengiz, 2003). Type of amino acids may affect growth, sporulation, and crystals of *B. thuringiensis* or vice versa. For example, a low concentration of cystine or cysteine helps growth but with high concentration only vegetative growth is seen. If cystine or cysteine concentration is 0.15% without crystal production heat variable spores are seen. When concentration is high (0.25%) spores and crystals are inhibited. Also, glutamate shows reducing impact against cystine inhibition (Merluzzi & Johnson, 1974).

2.2.9. Effects of carbon, nitrogen and salt source on growth, sporulation and toxin production

B. thuringiensis production based on various nutrient sources and this interaction of process' variables affect growth quantity and quality. Especially balance of carbon and nitrogen ratio is important for preventing lower pH level 5.6. In one study it was shown that C:N ratio 7:1 generated higher growth yield and glucose consumption with *B. thuringiensis* HD-73 strain (Betz et al., 2000; Torre, 1998) Glucose concentration is

another important medium pattern of *B. thuringiensis*. High glucose concentration (50-90 g/l) indicated that lower spores, crystal protein and showed lower toxicity against *Spodoptera littoralis* and *Anagasta kuhniella* larvae. The best result supplied with 20 g/l concentration of glucose. But only 78.3% of glucose is converted into spore and crystal formation. Rest of them are converted into by-products such as acetic acid and B-hydroxybutric acid (Amin, Alotaibi, Narmen, & Saleh, 2008).

Comparison of different medium for Cry1Ac protoxin production from HD73 indicated that ½ LB (Luria-Bertani) medium was the ideal medium for crystal production (112.38) in 300 mL LB. Also, repeated crystal solubilization method was a better way to Cry protoxin preparation (Zhou et al., 2015). As a supplement material to LB medium raw potato flour trial by solid state fermentation (SSF) indicated that, this conditions could be enhanced production of endotoxin by *B. thuringiensis* subsp. *kurstaki* with in short gestation time without losing its toxicity. Agricultural raw materials with SSF method may become a cheap and efficient way to control of insects (Smitha, Jisha, Pradeep, Josh, & Benjamin, 2013).

Also, food barley fermentation media enable to bioinsecticide with low-cost production especially for HD-1 strain. In industrial scale production soybean or groundnut are used as nitrogen source, starch or hull-less barley are used as carbon source (Shojaaddini, Moharramipour, Khodabandeh, & Talebi, 2010). Wastewater sludge culture medium includes nutrients such as carbon, nitrogen, phophorus which are required for *B. thuringiensis* growth and sporulation condition. Results showed that semi-solid wastewater sludge is an alternative raw material but medium needs other protein sources (Syazwanee, Noormasshela, Azwady, Rusea, & Muskhazli, 2016). Mustard-seed meal medium allowed toxin production and growth of *B. thuringiensis* HD-73 strain. But this strain can be harvested after 72 h of fermentation. In all strains, spores and crystals were started to form after 24 h fermentation. 69 to 72 hours of fermentation the highest number of spore-crystal complex was seen (Gangurde & Shethna, n.d.). Again, another study showed that the combination of 20% molasses, 10% corn step liquor and distilled water medium with HD73 strain generated best spore and colony count number (Amin et al.,2016).

The combination of crude gruel and fishmeal was found effective for growth, sporulation and delta endotoxin production. Same researchers investigated that effect of diluted sea water as a source of minerals with soya bean (nitrogen source) and yeast extract concentration. When using 25 g/l soya bean gives the highest delta endotoxin production. With this soya bean concentration (25 g/) and 30 g/l starch, four folds diluted sea water improved biomass production (Dhouha Ghribi, Zouari, Trabelsi, & Jaoua, 2007).

Apart from the effect of all these carbon, nitrogen sources and parameters, potassium (K_2HPO_4) and metal ions essential for spore production due to the stres requirement of spores. Metal ions include; Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} . These are very important for the highest sporulation and δ-endotoxin production (Içgen et al., 2002).

2.3. *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae)

The Tomato Leaf Miner, *Tuta absoluta*, is one of the most detrimental pest of tomato and other vegetables such as potato, eggplant, pepper, bean etc. Especially, tomato plants are infested with *T. absoluta* both outdoor and greenhouse conditions, and this pest devastating tomato production seriously all around the world (EPPO, 2005).

Figure 5 Adult Tomato leaf miner *(Tuta absoluta)*
(Anonymous)

2.3.1. Biology

T. absoluta life-cycle consist of four stages: egg, larva, pupa and adult. Adults lay eggs on the underside of leaves or stems neraly 250 to 300 in number (Cherif, Mansour, & Grissa-lebdi, 2013; Desneux et al., 2010) After hatching, first stage larvae penetrate leaves, fruits or stems and they feed with these parts of plant. There are four larval stages. After last larval stage, pupation take place on leaves or soil generally. Pupae length are 5–6 mm cylindrical shape and when they are near emergence an adult its color is increasingly become darker. Adults are 6–7 mm in length silver to grey coloration. This pest shows nocturnal habits. Adults usually remain hidden all day long among the leaves (Urbaneja, González-Cabrera, Arnó, & Gabarra, 2012). Tomatoes (*Lycopersicon esculentum* Miller) is to be the primary host of *T. absoluta*. Developmental stages depend on environmental conditions, nearly 23.8 days at 27.1℃ and generally ranged between 10-15 days for females. The larvae can cause 80 to 100% losses in tomato if it is not controlled (Birhan, 2018).

Figure 6 Mines of *Tuta absoluta* on tomato leaf (http://www.nbair.res.in/insectpests/Tuta-absoluta.php)

2.3.2. Ecology and Prevalance

It is first discovered from Peru in 1917 and identified as leafminer of tomato (*Solanum lycopersicum L.*) (Guedes & Picanc, 2012). The pest has invaded quickly more than 30 Western countries after the first detection on tomato in Spain 2006 and then

become a serious threat on world tomato production (Desneux et al., 2010; Roditakis et al., 2013)

In 2004, *T. absoluta* was added by the European and Mediterranean Plant Protection Organization (EPPO) to the A1 list of pests and then transferred to the A2 list in 2009.

The pest has spread to Ethiopia in 2012 presumably from Sudan or Yemen and South Africa in 2016 (Visser, Uys, Nieuwenhuis, & Pieterse, 2017). This pest spread to worlwide through the natural dispersal vehicle, such as wind (Gontijo et al., 2013), and fruit and vegetable transportation processes (Karadjova, Ilieva, Krumov, Petrova, & Ventsislavov, 2013).

The Food and Agriculture Organization of the United Nations (FAO) reported that about 177 million tonnes of tomato are produced worldwide (FAOSTAT, 2016). Tomato has become one of the most widely cultivated crops (more than 4.5 million hectares) worldwide. Because of this economic importance of tomato production, *T. absoluta* and its agricultural control have become important in worlwide.

2.3.3. Damage

T. absoluta is a detrimental pest on tomato. It attacks leaves, stems and fruit and creates mines and galleries on them during larval stage which reduces the yield of production (Estay 2000). Also they negatively affect photosynthetic capacity. Galleries in stems cause necrosis and when they are in fruits, plants can be invaded by secondary pathogens. The larvae can cause 80 to 100% losses in tomato if it is not controlled (Birhan, 2018).

T. absoluta females would prefer leaves in order to lay on egg particularly apex part than the other parts of the plant (Cherif et al., 2013). This oviposition behaviour may be related with a lower calcium content of apical plant leaves. The oviposition of *T. absoluta* was generated by volatile signals which is arise from their host plant such as terpenoid. This volatile compound attracts mated females (Proffit, Birgersson, & Bengtsson, 2011).

2.3.4. Damage and loss of production in Turkey

Turkey is the 4th tomato producers in the world with 11 million tons per year. The tomato, which accounts for approximately 65% of the fresh fruit and vegetable exports of countries, provides an input of approximately 400 million US dollars per year according to the 2013 data. *T. absoluta* is one of the major pest groups in the quarantine lists, which constitute significant economic losses on tomato in Turkey. In 2011, according to prevalance study of *T. absoluta*, highest infestation rates were found in provinces of Hatay (100%) and Osmaniye (100%), Adana (95.41%), Mersin (76.43%), Gaziantep (72.55%), Kahramanmaras (30.67%) and Kilis (1.27%) in Eastern Mediterranean and Southeastern Anatolia (Lepidoptera & Eastern, 2013). Besides these, Antalya, Aydın, Balıkesir, Burdur, Bursa, Çanakkale, Denizli, Düzce, Eskişehir, Gaziantep, Hatay, Iğdır, İzmir, Karaman, Kayseri, Konya, Kütahya, Manisa, Muğla, Samsun, Şanlıurfa, Tekirdağ, Tokat, Uşak and Yalova provinces have been shown to be contaminated (Birhan, 2018; Gelechiidae, 2011)

Control strategies of *T. absoluta* is widely carried out with chemical insecticides. However, in recent years, *T. absoluta* has developed different resistance mechanisms against these chemical agents in both research and field studies (Sansinenea, Salazar, Ramirez, & Ortiz, 2015; Siqueira, Guedes, & Picanço, 2000). The development of resistance mechanisms leads to more use of these chemicals and consequently to residual problems on tomatoes (Fe, Aires, & Aires, 2005). These remains lead to the return of the products after export from the customs. Because of the negative effects of chemical insecticides on the environment, natural life, water and human health , the use of biological agents instead of chemical pesticides is an important strategy (Bravo et al., 2011).

Except chemical insecticides and *B. thuringiensis* strains in the contol of *T. absoluta*; potential natural enemies, insect parasitoids (Desneux et al., 2010; Sabbour & Soliman, 2014; Zappalà et al., 2013) azadirachtin like plant extracts (Durmuşoğlu, 2011), entomogenous fungal strains (Sabbour & Soliman, 2014) and their secondary metabolites pheromone-based management strategies (Caparros Megido, Haubruge, & Verheggen, 2013) have been used.

2.3.5. Control Strategies

Biological control, especially the use of *B. thuringiensis* species and the formulations obtained using this bacterium are more effective on the control of *T. absoluta* (Hafsi, Abbes, Chermiti, & Nasraoui, 2012; Salem, 2013). *B. thuringiensis* has been used in commercial and academic studies with different bacterial variants for a long time (Höfte & Whiteley, 1989). Bacillus-based commercial formulations such as RAPAX, DIPEL DF, FLORBAC WG, BIOBIT WP, BIO-T PLUS, REBOUND WP and AGREE WG have been widely used in Turkey. However, these products are obtained through importation and they are not locally produced (bku.tarim.gov.tr).

Licensed *B. thuringiensis* containing products are intended to have a common effect by incorporating specific toxin species from their Cry1 and Cry2 families. *B. thuringiensis* HD1 strain, which was used in the first studies and formulations, was shown to be more effective than commercial products and later it was shown that the HD73 strain was more effective than HD1 formulation (H. T. Dulmage et al., 1971). Similarly, the strain HD73 was found to be more effective than the mixed commercial product (Dipel Df) on *T. absoluta* (Sabbour & Soliman, 2014).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Preparation of Laboratory Materials

All glassware, media, solutions, micropipette tips and microcentrifuge tubes were sterilized at 121℃ for 15 minutes before use. Glasses also were washed with detergents and rinsed with distilled water before autoclaving.

3.1.2. Solutions

- 4X Separating Buffer (1.5 M Tris, pH 8.8, 0.4% SDS)

  -Tris base 36.34 g

  -SDS 0.8 g

  Dissolved in 200 ml deionized water, pH was adjusted to 8.8 with 6M HCl, stored at 4°C.

- 4X Stacking Buffer (0.5 M Tris, Ph 6.8, 0.4% SDS)

  -Tris base 6.04 g

  -SDS 0.4 g

  Dissolved in 100 ml deionized water, Ph was adjusted to 6.8 with 6M HCl, stored at 4℃.

- 10% SDS Solution

  -SDS 10 g

  Volume was completed to 100 ml with deionized water. Stored at room temperature.

- 10% Ammonium persulfate solution

  -APS 0.5 g

  Volume was completed to 5 ml with deionized water. Aliquoted and stored at -20˚C.

- 10X Running buffer

  -Tris base 15 g

  -Glycine 72 g

  -SDS 5 g

  Volume was completed to 500 ml with deionized water. It was stored at room temperature and diluted to 1X before used.

- 4X Sample loading buffer

  -4X Stacking 2.5 ml

  -SDS 1 g

  -Glycerol 4 ml

  -2-mercaptoethanol 2 ml

  -0.1% Bromophenol blue 0.8 ml

  pH was adjusted to 6.8 before adding bromophenol blue. Volume was completed to 10 ml with deionized water. Aliquoted in 1 ml microcentrifuge tubes and stored at -20°C in the dark.

- Bradford Reagent (1X)

  -BIO-RAD Quick Start Dye Reagent

- Staining Solution

  -Methanol 100 ml

  -Coomassie brilliant blue R-250 0.2 g

  -Acetic acid 20 ml

  Volume was completed to 200 ml with deionized water. Stored at room temperature.

- Destaining Solution

  -Methanol 25 ml

  -Acetic acid 35 ml

  Volume was completed to 200 ml with deionized water. Stored at room temperature.

3.2. Methods

3.2.1. Bacterial Strain and Culture Conditions

Bacillus thuringiensis subsp. *kurstaki* HD-73 and HD-1 strains obtained from American Type Culture Collection (ATCC).

HD-73 strain was regularly maintained on Nutrient agar (beef extract 3.0 g, peptone 5.0 g, agar 15.0 g per 1000 ml distilled water) at 30℃.

HD-1 strain was maintained on Tryptic soy agar (tryptone 17,0 g, soytone 3,0 g, dextrose 2,5 g, NaCl 5,0 g, K2HPO4 2,5 g, agar 15,0 g per 1000 ml distilled water) at 30°C.

Strains were subcultured from main stock on their designated agar media. Then single colonies were selected and after 16 hours of growth in liquid media they were stored in 30% glycerol in -80℃ freezer for long term storage.

Table 2 Bacterial strains and culture media

Strain	ATCC Code Number	Medium
HD73	60033905	Nutrient Agar / Broth
HD-1	63372696	Tryptic Soy Agar/ Broth

3.2.2. Media Preparation

To optimize growth and toxin production various growth media containing different carbon, nitrogen and salt sources and concentrations were used (Table 3.2). A pH range was also assayed in selected growth media (Nutrient broth). Molasses, whey powder, yeast extract and peptone were used as carbon and nitrogen sources; and Mn^{2+}, Ca^{2+}, Mg^{2+} were used in different concentration as salt sources. All media that were used in experiments are given in Table 3. All media were prepared using ddH2O, pH adjusted and autoclaved at 121℃ for 15 min before use.

Table 3 Growth media containing varying carbon sources and salt contents used in optimization studies.

Media	Content	Salts
Nutrient Broth (NB)	Beef extract 3.0 g/L Peptone 5.0 g/L	----
Nutrient Agar (NA)	Beef extract 3.0 g/L Peptone 5.0 g/L Agar 15.0 g/L	----
Nutrient Yeast Salt Medium (NYSM)	Beef extract 3.0 g/L Peptone 5.0 g/L Yeast extract 0,5 g/L	5 ml salt mixture; $CaCl_2.2H_2O$ 10.0 g/L $MgCl_2.6H_2O$ 20.35 g/L $MnCl_2.4H_2O$ 1.0 g/L
(TBL)	Beef extract 3.0 g/L Peptone 5.0 g/L Yeast extract 0,5 g/L Bacto peptone 7.5 g/L Glucose 1.0 g/L K_2HPO_4 4.35 g/L KH_2PO_4 3.4 g/L	5 ml salt solution $MgSO_4.7H_2O$ 24.6 g/L $MnSO_4.H_2O$ 0.4 g/L $ZnSO_4.7H_2O$ 2.8 g/L $FeSO_4.7H_2O$ 4 g/L 5 ml of $CaCl_2.2H_2O$ 36.6 g/L solution

Molasses Medium (MM) *[Molasses Salt Medium (MSM)]*	Molasses 20,0 g/L	When necessary; 5 ml salt mixture; $CaCl_2.2H_2O$ 10.0 g/L $MgCl_2.6H_2O$ 20.35 g/L $MnCl_2.4H_2O$ 1.0 g/L
Molasses Beef Medium (MBM) *[Molasses Beef Salt Medium (MBSM)]*	Molasses 20,0 g/L Beef extract 3.0 g/L	
Molasses Peptone Medium (MPM) *[Molasses Peptone Salt Medium (MPSM)]*	Molasses 20,0 g/L Peptone 5.0 g/L	
Molasses Beef Peptone Medium (MBPM) *[Molasses Beef Peptone Salt Medium (MBPSM)]*	Molasses 20,0 g/L Beef extract 3.0 g/L Peptone 5.0 g/L	
Molasses Yeast Medium (MYM) *[Molasses Yeast Salt Medium (MYSM)]*	Molasses 20 g/L Yeast extract 5,0 g/L	
Beef Peptone Yeast Whey Medium (BPYWM) *[Beef Peptone Yeast Whey Salt Medium (BPYWSM)]*	Beef extract 3.0 g/L Peptone 5.0 g/L Yeast extract 0,5 g/L Whey powder 0,5 g/L	
Beef Peptone Whey Medium (BPWM) *[Beef Peptone Whey Salt Medium (BPWSM)]*	Beef extract 3.0 g/L Peptone 5.0 g/L Whey powder 0,5 g/L	
Peptone Yeast Whey Medium (PYWM) *[Peptone Yeast Whey Salt Medium (PYWSM)]*	Peptone 5.0 g/L Yeast extract 0,5 g/L Whey powder 0,5 g/L	
Whey Yeast Medium *[Whey Yeast Salt Medium (WYSM)]*	Whey powder 8,0 g/L Yeast extract 0,5 g/L	

3.2.3. Inoculum

In all experiments, cultures were prepared as described in here. Appropriate agar plates were streaked from -80℃ storage and incubated for 16 hours at 30℃. Then at least 3 single colonies were used to inoculate a culture tube containing 6 ml appropriate growth medium. Inoculated culture tubes were then incubated for at least 16 hours at 30℃ with 250 rpm constant shaking. Later 50 ml of selected fresh medium in a 250 ml flask was inoculated from this o/n culture with 1/100 dilution and incubated at 30℃ for 72 hours with 250 rpm constant shaking.

3.2.4. Growth Curve

50 ml of medium in 250 ml flask was inoculated with 1/100 dilution of o/n grown Btk-HD73 and incubated for 72 hours 30℃ with 250 rpm shaking. During incubation 100

µl of samples were taken to 1 ml microcentrifuge tubes at predetermined time points. Samples were then diluted (1/10) with sterile 0.9% NaCl and vortexed. From this dilution 200 µl samples were transferred into 3 microplate wells. Cell density was measured by taking absorbance values at 600 nm using a spectrophotometer. Absorbance of 0.9% NaCl was used for blank calculation. Growth curve was plotted using Microsoft Excel Software in MS Windows environment.

3.2.5. Spore Count

During the incubation of Btk-HD73 in an assay medium, 10 ml of samples were collected at 24th, 48th and 72nd hours. Samples were then centrifuged at 7.500 rpm for 15 minutes at 4℃, supernatant was removed and then washed with 2 ml of 0.9% NaCl. This washing stage was repeated twice and after the last washing step the obtained pellet was stored at 4℃ until used. At 72nd hour point incubation was terminated, and all medium was centrifuged at 7.500 rpm for 15 minutes at 4℃. After spore count, this pellet was used for entomotoxicity assays on *Tuta absoluta*.

For spore count, the following method was used (Shojaaddini et al., 2010). Stored pellet samples were dissolved in 2 ml 0.9% NaCl. From this solution, 100 ul of sample was taken and mixed with 900 ul of 0.9% NaCl and vortexed with glass beads. Then this mixture was agitated in a shaker at 1.000 rpm for 15 min. Homogenized samples were then incubated at 80℃ for 15 minutes. Serial dilutions were plated on nutrient agar plates with at least three replicates and incubated at 30℃ for at least 16 hours to obtain visual colonies. Btk-HD73 colonies were counted and reported as CFU/ml.

3.2.6. Phase Contrast Microscopy

The presence of spore and crystal protein was observed by Phase contrast microscopy. Every 24 hours, a drop of sample was taken from flask and was placed on a microscopy slide. Samples were examined at 100X magnification and corresponding images recorded for every sample.

3.2.7. Delta-endotoxin determination

Delta endotoxin concentration was determined in the solubilized crystal preparation from each culture medium as described by Zouari et al. (1998). To obtain

crystal preparation, 1 ml of culture medium was centrifuged for 5 minutes at 10,000 x g and the pellet was washed twice with 0.9% NaCl. The pellet was then resuspended in 1 ml of 50 mmol/l NaOH (pH 12.5) to solubilize delta-endotoxin crystals. After 2 hours of incubation at 30℃, total proteins in the supernatant was measured by using Bio-Rad Protein Assay Kit (Cat. no 5000001) according to the manufacturer protocol based on Bradford method (1976) as described below. The toxin synthesis yield was calculated as the ratio of delta-endotoxin (mg/l) relative to CFU/L (spores/l).

3.2.7.1. Bradford Assay

To obtain a standard curve, diluted concentrations of BSA standards (2.0, 1.5, 1.0, 0.75, 0.5, 0.25, 0.125 mg/ml) were prepared in microcentrifuge tubes. Samples containing toxins were diluted with NaOH at the rate of 1:2, 1:4, 1:8 and 1:16 in microcentrifuge tubes.

One ml of 1X dye reagent was added to each tube and vortexed. After 30 minutes of incubation at room temperature, 200 µl from each sample was transferred to a 96-well microplate well and absorbance value was taken at 595 nm using a microplate reader.

A standard curve of absorbance versus protein concentration was obtain using BSA standards. The amount of protein in supernatant was calculated from that standard curve with the following equation.

y=mx, where:

y is the absorbance at 595 nm

m is the slope of the standard curve

x is the protein concentration in mg/ml

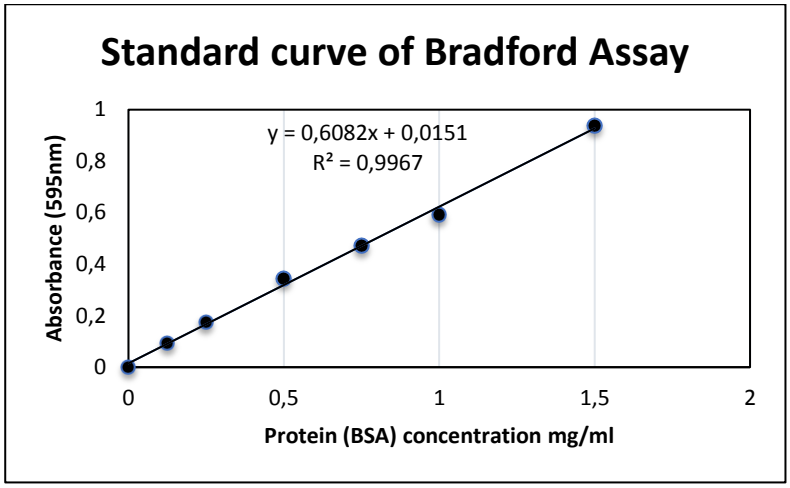


Figure 7 A representative standard curve for estimation of protein concentration by Bradford method

3.2.8. SDS-PAGE Analysis of Cry1Ac protoxin

Cry1Ac protoxin (133.3-kDa) was visualized by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). Electrophoresis was performed at 75 mA for 30 minutes and at 100 mA for 60 minutes. Gels were stained with Coomassie Brilliant Blue for 45 minutes at room temperature and destained in glacial acetic acid 6.75% (v/v) and methanol 9.45% (v/v) for 60 minutes at room temperature.

3.2.8.1. Sample Preparation

One ml of sample from growing cultures was taken into microcentrifuge tubes, centrifuged at 10.000 rpm for 5 minutes and obtained pellet was washed twice with 0.9% NaCl. Pellets were then stored at -80℃ till used.

Stored samples were resuspended in 60 µl of SDS sample buffer boiled for 5 minutes on a conventional heating block at 90℃ and centrifuged for 10 minutes at 10,000 rpm. Then 5 ul from the supernatant was loaded onto SDS-PAGE in gel slabs of 1.00 mm thickness (8% separating and 4% stacking gels) as described by Laemmli (1970).

3.2.8.2. Page Preparation

8% Separating gel was prepared by mixing distilled water (4.6 ml), 4X separating buffer (2.6 ml), 30% acrylamide-bisacrylamide solution (2.6 ml), 10% SDS (100 µl), 10% ammonium sulfate (APS) (100 µl) and rapid addition of TEMED (10 µl).

4% stacking gel was prepared by mixing the distilled water (3.05 ml), 4X stacking buffer (1.25 ml), 30% acrylamide-bisacrylamide solution (0.65 ml), 10% SDS (50 µl), 10% ammonium sulfate (APS) (25 µl) and rapid addition of TEMED (5 µl).

Separating gel mix was poured into glass plate chamber leaving 3 cm from the top of the glass plate to add stacking gel later. To remove air bubbles and fix the gel, 2-propanol was poured onto the gel. After the solidification of the separating gel for about 20 minutes, 2-propanol was removed by rinsing with distilled water. Then stacking gel solution was poured on top of separating gel. Later a 10-well comb was placed on top and the gel was allowed to polymerize for at least 20 minutes. After polymerization the comb was removed.

3.2.8.3. Sample application and electrophoresis run

Gel cassette was placed into the BIO-RAD Mini-Protein Tetra cell system containing running buffer (1X). Then samples (5 µl) and protein markers were loaded into wells. Electrophoresis was carried out at 100 V for approximately 120 minutes.

3.2.8.4. Staining and destaining of the gel

Gel was incubated in the staining solution (0.02 Coomassie Brilliant Blue G-250 in 2% phosphoric acid, 5% aluminum sulfate and 10% ethanol) for 45 minutes at room temperature with constant shaking at 50 rpm using a rotary shaker. The gel was then washed thrice for 20 minutes in destaining solution (7% acetic acid and 5% methanol) again with constant shaking at 50 rpm.

Bands were screened and the image was captured using a gel documentation system (BIO-RAD Gel Doc EZ Imager).

3.2.9. Optimization of bioreactor process

In the 4.5-liter bioreactor vessel 3 liters of selected growth medium was used. Media compositions were given in Table 3. The pH electrode was calibrated using pH7 and pH4 buffers before the sterilization. Bioreactor vessel containing the selected medium was autoclaved at 121℃ for 20 minutes and cooled to 30℃ prior to use. Then, 15 ml (1% v/v inoculum) of actively growing Btk-HD73 was inoculated to medium. All inoculations were made using 15 ml (15 v/v) of actively growing Btk-HD73 nutrient broth culture previously inoculated using a single colony and incubated for 16 hours at 30℃.

In bioreactor process, dissolved oxygen, pH, impeller speed, temperature and aeration rate were controlled by an automatic control system. Temperature was set at 30°C±1 and controlled by a circulating water chiller unit. pH was kept constant within a range of 7.0±0.2 using 1N H_3PO_4 (phosphoric acid) and 1N NaOH (Sodium hydroxide) administered when necessary via peristaltic pumps. The agitation speed was set at 250 rpm. The foam was controlled by adding oleic acid when needed.

Samples were periodically collected every 24 hours to determine total cell count, spore count, toxin expression, microscopic analyses and entomotoxicity via bioassays.

3.2.10. Bioassay

3.2.10.1. Cultivation of tomato plants

Tomato plants were grown in greenhouse condition (Konya Food & Agriculture University Research Greenhouse).

3.2.10.2. Rearing insect pests

T. absoluta were reared on tomato leaves in rearing cages, under laboratory conditions. Temperature was maintained at 25℃ and humidity (RH) within the range of 50-60%.

3.2.10.3. Effect of *Bacillus thuringiensis* subsp. *kurstaki* HD-73 and Dipel on *Tuta absoluta*

Btk-HD73 spores were harvested from selected growth media. These samples were prepared in 3 concentrations (X/2, 1X, 10X) based on gi

ven spore number of the commercial formulation (Dipel). In all conditions same spore numbers were used in 100 ml distilled water.

Tomato leaves were dipped in solutions containing known numbers of spores (Btk-HD73 or Dipel) and put in plastic cups to dry. For each concentration, three leaves were assayed (triplicate). Four larvae of *T. absoluta* (2^{nd} instar) were put on tomato leaves and covered with plastic foil. The experiments were carried out in incubator at 25℃. Control group included larvae on untreated leaves. Commercially available Dipel was used as a positive control.

After 3 days, the percentages of mortality were calculated according to Abbott, 1925. Also LC_{50} was calculated with probit analysis (Finney,1964).

3.2.10.4. Calculation of LC_{50} value

The LC_{50} value, 95% confidence limits, standard errors, the slopes of the regression lines and $\chi 2$ signifcance tests, were estimated by probit analysis using PoloPlus 1.0 software (LeOra Sofware, California, United States).

4. RESULTS

4.1. Conventional culture studies of *Bacillus thuringiensis* subsp. *kurstaki* HD-73 and optimization of toxin production

4.1.1. Colony characteristics

Nutrient agar plates were streak from -80℃ stocks of Btk-HD73 and incubated at 30℃ for at least 16 hours. On nutrient agar plates colonies were white in color, opaque and slightly raised (Figure 8).

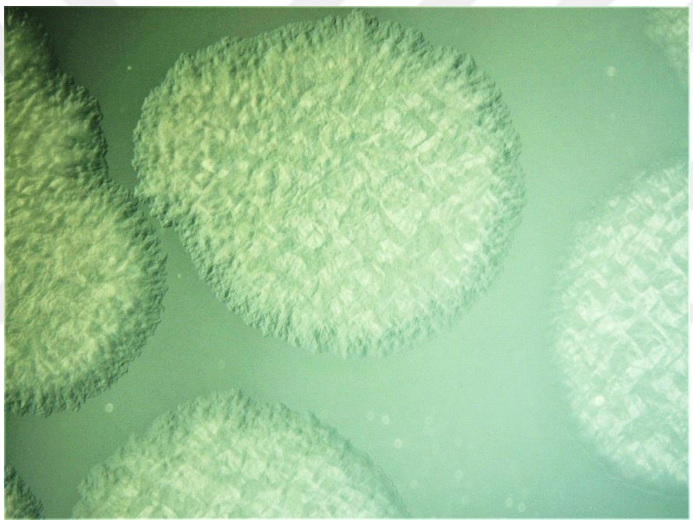


Figure 8 Colonies of Btk-HD73 on nutrient agar plates.

4.1.2. Spore morphology and crystal toxins

Samples were taken from a 72-hours old NYSM incubated at 30℃ with 200 rpm constant shaking. Collected samples were visualized using a phase contrast microscope at 100X magnification.

Spores and crystal toxins can be identified as green appearances on the dark background (Figure 9).

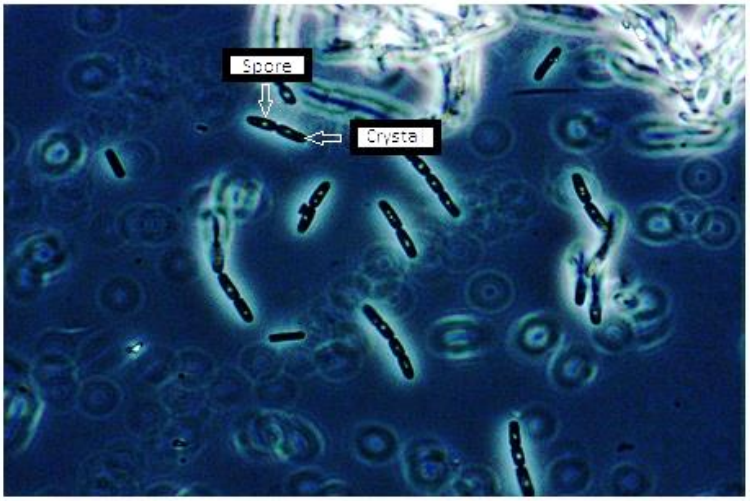


Figure 9 Spore morphology and crystal toxin proteins of Btk-HD73 under phase contrast microscope.

4.1.3. Growth curve of HD-73 and HD-1 in conventional media

HD-73 and HD-1 strains exhibited a similar growth trend in conventional NB medium (Figure 10). They entered into exponential growth phase approximately 2 hours after the inoculation and reached stationary phase 6 hours later. It was concluded that maximum cell number could be achieved 10 hours after inoculation under our conditions tested.

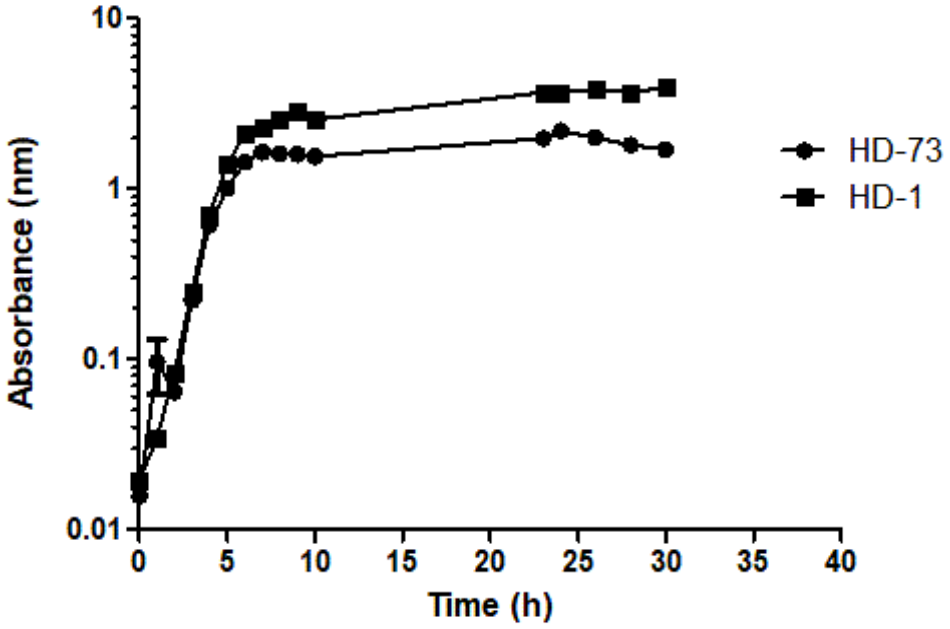


Figure 10 Growth curves of Btk-HD73 and HD-1 strains in nutrient broth.
Cultures were incubated at 30℃ with 250 rpm constant shaking and cell density was measured by taking absorbance values at 600 nm.

For the purpose of this thesis work we continued our optimization studies with HD-73 strain only.

To observe spore formation Btk-HD73 was grown in NB at 30°C for 30 hours with 250 rpm constant shaking (Figure 11). Samples corresponding to late exponential (at 7^{th} hour) and late stationary (at 28^{th} hour) phases were taken and visualized under phase contrast microscope (Figure 12).

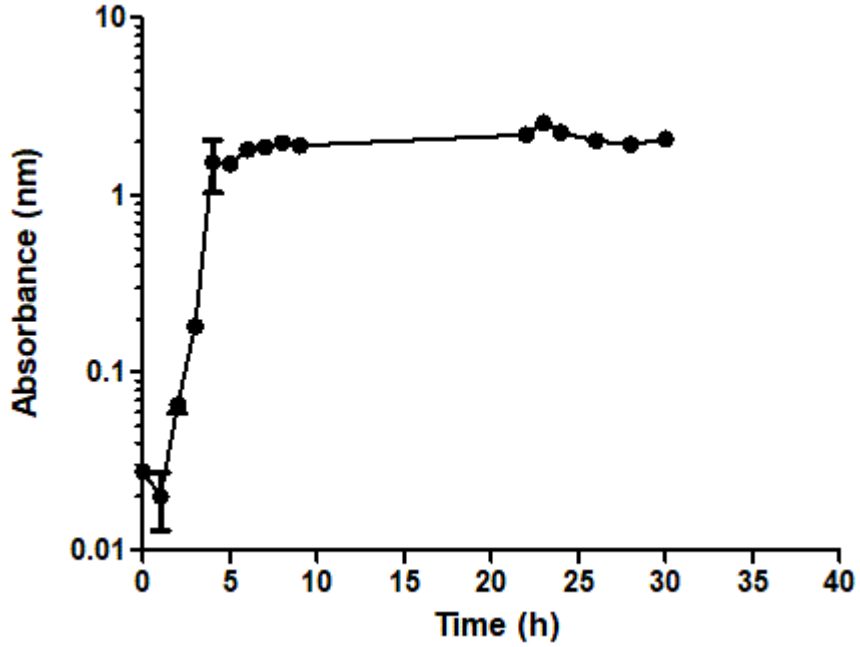


Figure 11 Growth curve of Btk-HD73 in nutrient broth.
Culture was incubated at 30℃ with 250 rpm shaking and cell density was measured by taking absorbance values at 600 nm.

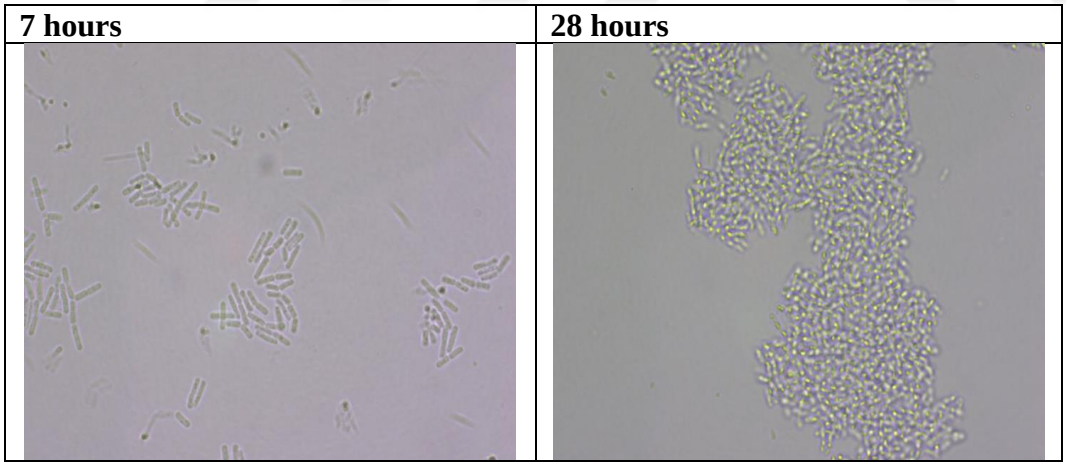


Figure 12 Phase contrast microscope images of Btk-HD73 at 7 and 28 hours in NB.
Culture was incubated in NB medium at 30 C with 250 rpm shaking for 30 hours. Sampling times corresponding to late exponential (at 7th hour) and late stationary (at 28th hour) phases.

Bacteria were in their vegetative form at 7th hour. As this was late exponential phase of the growth curve, almost no endospore could be observed at this time point. In

samples collected at 28th hour, spores were visible and were the majority with other vegetative bacteria in aggregates (Figure 12).

4.2. Screening of Cry1Ac Crystal Protein with SDS-PAGE

Btk-HD73's crystal toxin protein production was visualized at different time points using SDS-PAGE analysis during growth in NB at 30℃ with 250 rpm constant shaking (Figure 13).

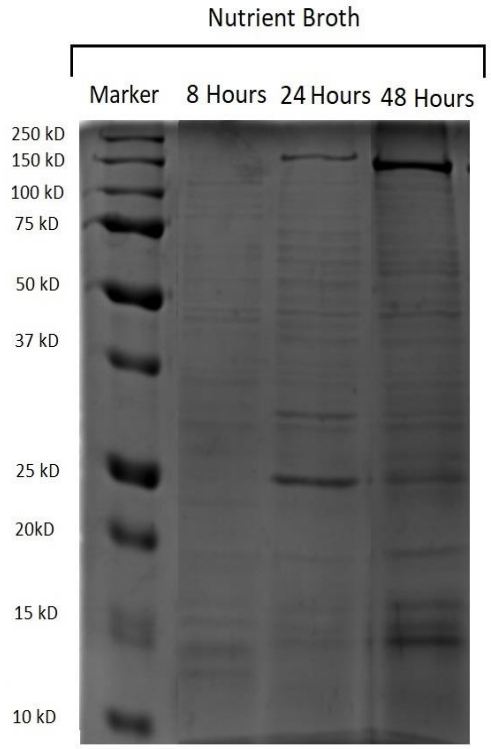


Figure 13 Btk-HD73 total protein SDS-PAGE analysis at 8th, 24th and 48th hours in NB.
Btk-HD73's crystal toxin protein production was visualized at 8th, 24th and 48th hour time points using SDS-PAGE analysis during growth in NB at 30℃ with 250 rpm constant shaking.

There was no visible 133kD crystal toxin protein band in 8th hour sample. This time point falls in to the late logarithmic growth phase. A band corresponding to 133kD appeared in 24th and 48th hour samples with an increasing intensity (Figure 13). This result suggested that Cry1Ac toxin protein production in Btk-HD73 showing an increase in parallel to spore formation (Figure 12) in NB at conditions tested.

4.3. Effects of Different Carbon and Salts Sources on Btk-HD73's Growth, Spore Formation and Toxin Production

To assess the effect of carbon and salt sources on Btk-HD73's growth, spore formation and toxin production, growth media containing varying carbon sources and salt contents (Table 4) were used.

Table 4 Growth media containing varying carbon sources and salt contents used in optimization studies.

Media	Content	Salts
Nutrient Broth (NB)	Beef extract 3.0 g/L Peptone 5.0 g/L	----
Nutrient Agar (NA)	Beef extract 3.0 g/L Peptone 5.0 g/L Agar 15.0 g/L	----
Nutrient Yeast Salt Medium (NYSM)	Beef extract 3.0 g/L Peptone 5.0 g/L Yeast extract 0,5 g/L	5 ml salt mixture; $CaCl_2.2H_2O$ 10.0 g/L $MgCl_2.6H_2O$ 20.35 g/L $MnCl_2.4H_2O$ 1.0 g/L
(TBL)	Beef extract 3.0 g/L Peptone 5.0 g/L Yeast extract 0,5 g/L Bacto peptone 7.5 g/L Glucose 1.0 g/L K_2HPO_4 4.35 g/L KH_2PO_4 3.4 g/L	5 ml salt solution $MgSO_4.7H_2O$ 24.6 g/L $MnSO_4.H_2O$ 0.4 g/L $ZnSO_4.7H_2O$ 2.8 g/L $FeSO_4.7H_2O$ 4 g/L 5 ml of $CaCl_2.2H_2O$ 36.6 g/L solution
Molasses Medium (MM) *[Molasses Salt Medium (MSM)]*	Molasses 20,0 g/L	When necessary; 5 ml salt mixture; $CaCl_2.2H_2O$ 10.0 g/L $MgCl_2.6H_2O$ 20.35 g/L $MnCl_2.4H_2O$ 1.0 g/L
Molasses Beef Medium (MBM) *[Molasses Beef Salt Medium (MBSM)]*	Molasses 20,0 g/L Beef extract 3.0 g/L	
Molasses Peptone Medium (MPM) *[Molasses Peptone Salt Medium (MPSM)]*	Molasses 20,0 g/L Peptone 5.0 g/L	
Molasses Beef Peptone Medium (MBPM) *[Molasses Beef Peptone Salt Medium (MBPSM)]*	Molasses 20,0 g/L Beef extract 3.0 g/L Peptone 5.0 g/L	
Molasses Yeast Medium (MYM) *[Molasses Yeast Salt Medium (MYSM)]*	Molasses 20 g/L Yeast extract 5,0 g/L	
Beef Peptone Yeast Whey Medium (BPYWM) *[Beef Peptone Yeast Whey Salt Medium (BPYWSM)]*	Beef extract 3.0 g/L Peptone 5.0 g/L Yeast extract 0,5 g/L Whey powder 0,5 g/L	
Beef Peptone Whey Medium (BPWM) *[Beef Peptone Whey Salt Medium (BPWSM)]*	Beef extract 3.0 g/L Peptone 5.0 g/L Whey powder 0,5 g/L	

Peptone Yeast Whey Medium (PYWM) *[Peptone Yeast Whey Salt Medium (PYWSM)]*	Peptone 5.0 g/L Yeast extract 0,5 g/L Whey powder 0,5 g/L	
Whey Yeast Medium *[Whey Yeast Salt Medium (WYSM)]*	Whey powder 8,0 g/L Yeast extract 0,5 g/L	

4.3.1. Effect of molasses on sporulation and toxin production

We have first used molasses as an alternative carbon source to replace yeast or beef extracts used in the NB, which is the Btk-HD73's regular growth medium. The main idea behind this was to reduce the production cost as yeast or beef extract used in growth media are all imported and expensive; whereas molasses is easy to find especially in Konya region as it is the byproduct of sugar industry. Molasses is produced from sugar beet during the sugar production process and is currently being used in Turkey for different purposes as animal feed, fertilizer and biomass for ethanol production.

To this end, liquid media containing molasses of different proportions were prepared (Figure 14).

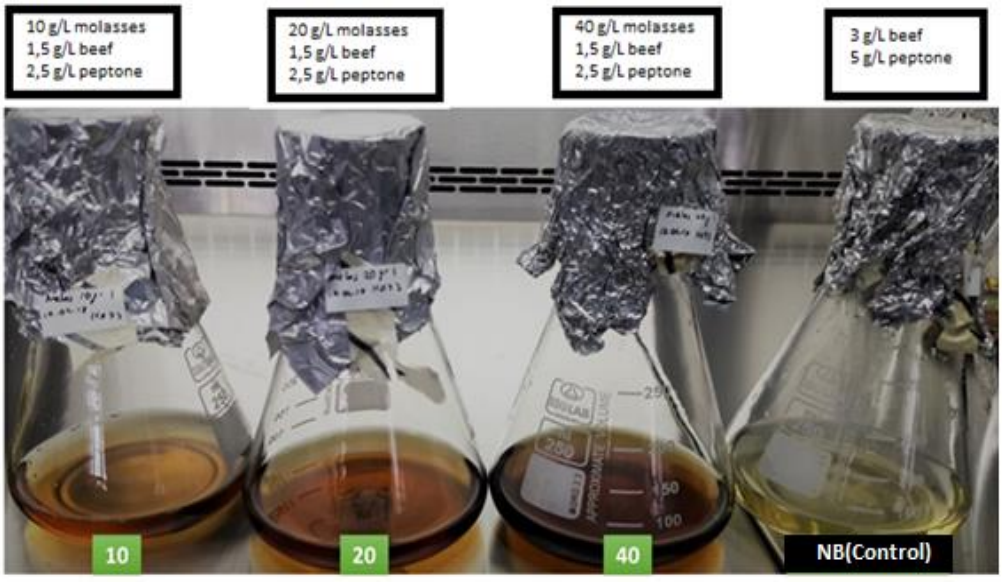


Figure 14 Media with different molasses content used in assays.
Contents of each medium are presented in the upper panel.

Btk-HD73 strain was inoculated in selected media and incubated for 72 hours at 30°C with 250 rpm constant shaking. Samples were taken from growing cultures and read

at 600 nm to construct a growth curve (Figure 15), and at 8th, 24th, 48th and 72nd hours samples were taken for SDS-PAGE analysis (Figure 16 and 17) and microscopic visualization (Figure 18, 19 and 20).

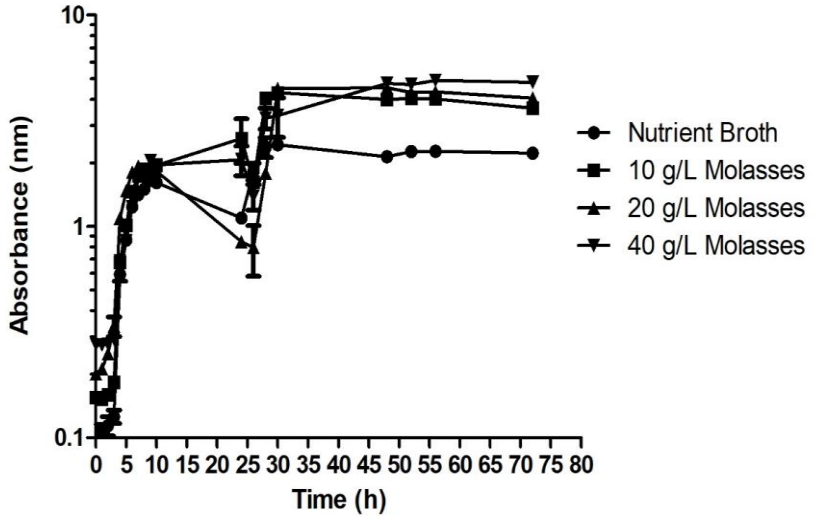


Figure 15 Growth curves of Btk-HD73 on liquid media containing molasses in different proportions.
Cultures were incubated at 30 ° C with 250 rpm constant shaking and the absorbance measurements were taken at 600 nm.

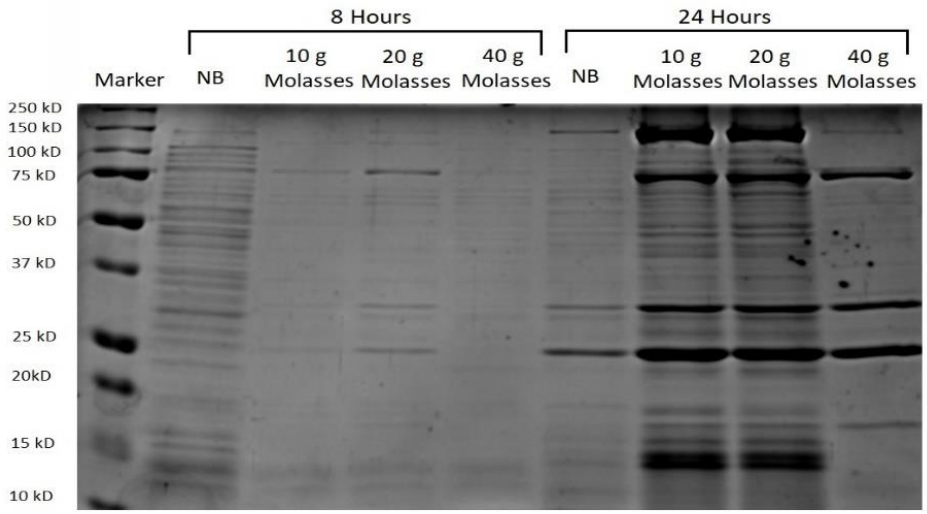


Figure 16 Btk-HD73 total protein SDS-PAGE analysis at 8th and 24th hours in media containing molasses.
The change in protein profile was observed by SDS-PAGE analysis at 8 and 24 hours depending on the growth of HD-73 strain in media containing molasses. The values next to the left column of the marker (M) are given in kDa.

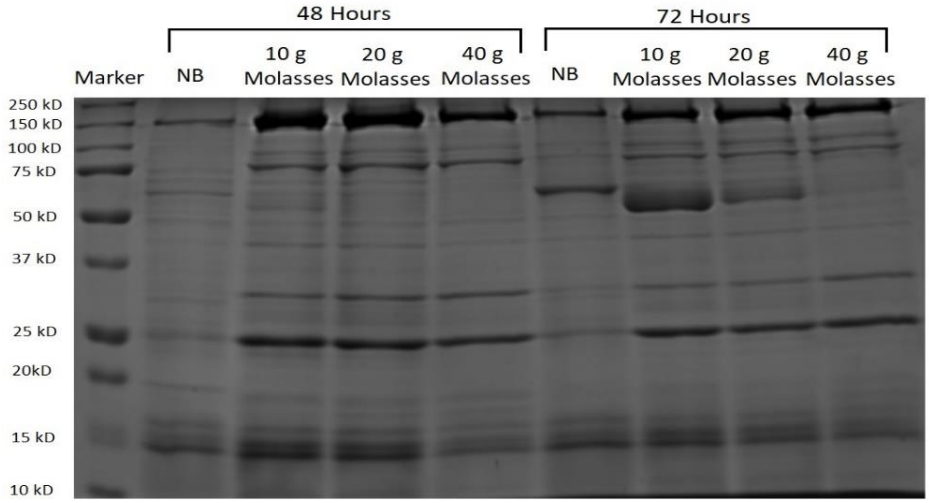


Figure 17 Btk-HD73 total protein SDS-PAGE analysis at 48th and 72th hours in media containing molasses. The change in protein profile was observed by SDS-PAGE analysis at 48 and 72 hours depending on the growth of HD-73 strain in media containing molasses. The values next to the left column of the marker (M) are given in kDa.

When the results of SDS-PAGE analysis were examined in parallel with the growth curve, it was observed that toxin production was similar for molasses concentrations of 10 and 20 g/L at 24th and 48th hours. In medium containing 40 g/L of molasses, toxin band was only visible in 48th hour sample, but not in 24th hour sample, and for this time point this band was less intense compared to media containing 10 and 20 g/L molasses. It was also noted that toxin protein production was higher in all molasses concentrations compared to NB for 24th and 48th hour time points.

At 48th hour 350 µl of salt mixture (10,15 g /L $MgCl_2$; 5,1 g/L $CaCl_2$; 0.5 g/L $MnCl_2$) was added to each medium after collecting 48th hour samples, and cultures were allowed to incubate for a further 24 hours under the same conditions. It was observed that the salt addition to cultures did not cause an increase in the amount of toxin production compared to 48th hour samples under these conditions tested. It was also noted that at 72nd hour, the amount of toxin protein production was roughly the same for all molasses concentrations.

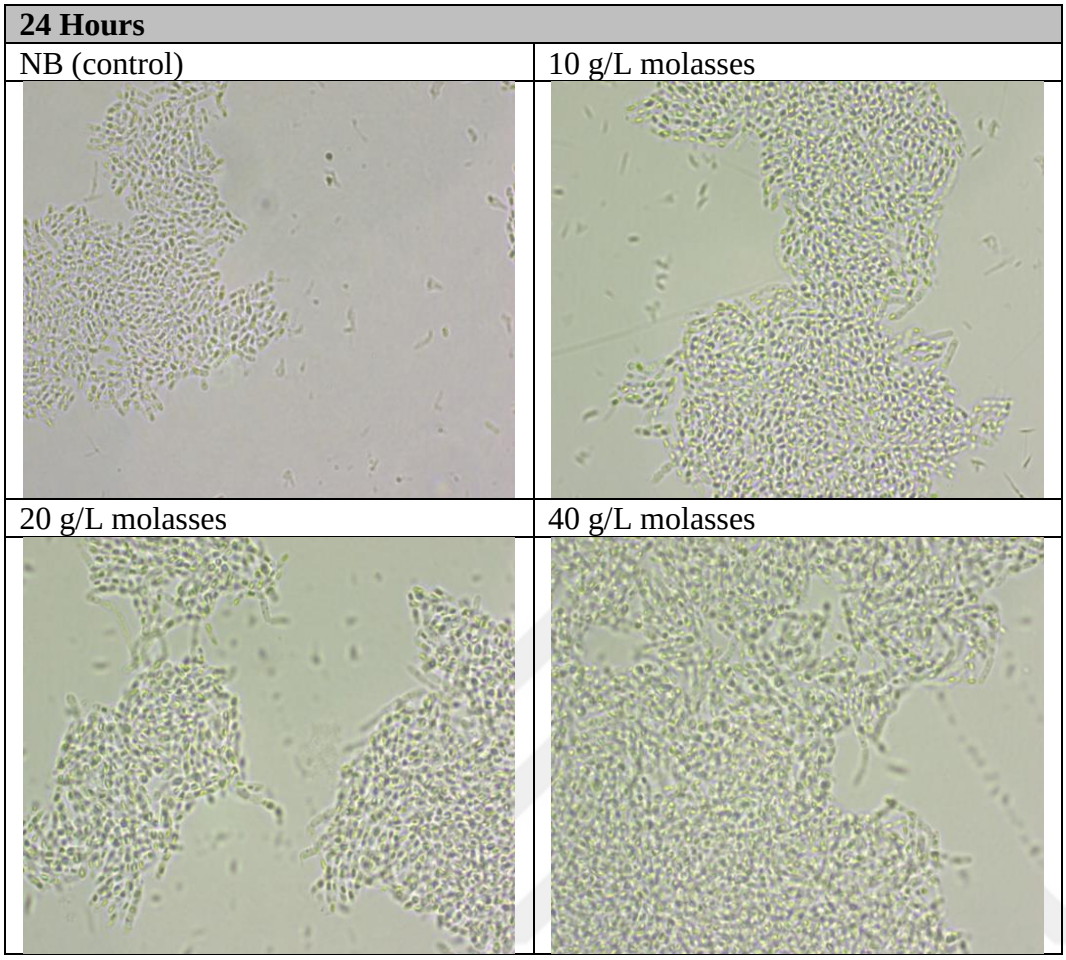


Figure 18 Phase contrast microscope images of Btk-HD73 at 24 hours in media containing molasses with varying concentrations.

Culture was incubated in liquid media containing molasses with varying concentrations at 30℃ with 250 rpm shaking for 72 hours . Samples were examined at 100X magnification under a phase contrast microscope at 24th hour.

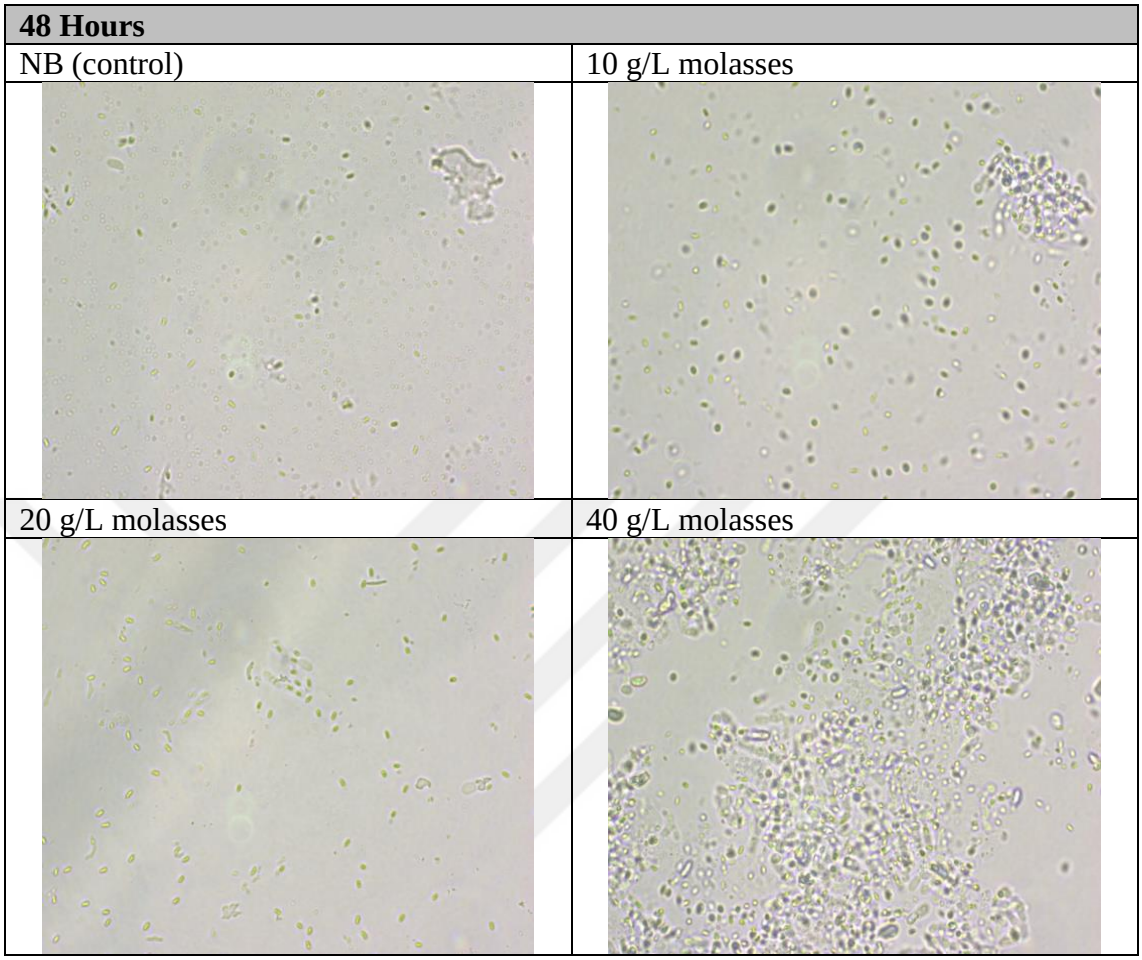


Figure 19 Phase contrast microscope images of Btk-HD73 at 48 hours in media containing molasses with varying concentrations.

Culture was incubated in liquid media containing molasses with varying concentrations at 30℃ with 250 rpm shaking for 72 hours. Samples were examined at 100X magnification under a phase contrast microscope at 48th hour.

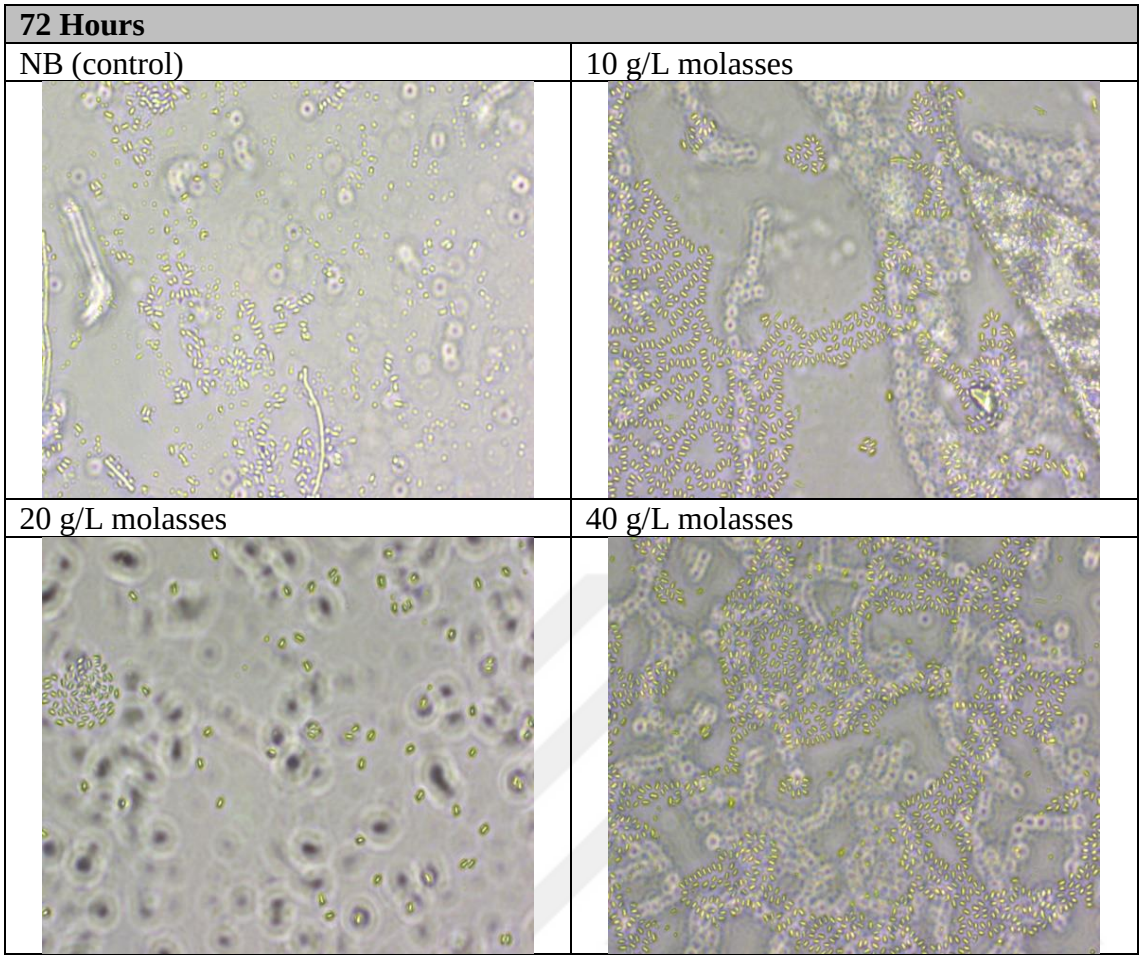


Figure 20 Phase contrast microscope images of Btk-HD73 at 72 hours in media containing molasses with varying concentrations.

Culture was incubated in liquid media containing molasses with varying concentrations at 30℃ with 250 rpm shaking for 72 hours. Samples were examined at 100X magnification under a phase contrast microscope at 72th hour.

For each time point samples were also visualized using a phase contrast microscope for the presence of spores. A linear correlation was observed between culture age and the spore numbers.

When the microscope images, growth curves and the results of SDS-PAGE analysis were evaluated together: **(1)** the addition of salt solution did not increase the toxin amount, but it caused an increase in spore numbers; **(2)** it was observed that molasses concentrations of 20 g/L and 40 g/L were suitable for sporulation in the presence or absence of salt. However, according to the SDS-PAGE results, it was found that molasses concentration of 20 g/L would be more suitable for toxin production; **(3)** it was observed that when molasses used as a carbon source there was no correlation between sporulation and toxin production in Btk-HD73.

According to these results, 20 g/L concentration of molasses as the carbon source was selected to be used in later optimization experiments where effects of other medium components such as yeast extract, beef extract on growth (Figure 21), toxin production (Figure 22, 23, 24 and 25) and spore production (Figure 26, 27 and 28) were investigated.

To this end growth of Btk-HD73 was first tested in media containing molasses and variations of peptone, yeast extract and beef extract as given in Table 4. NB was used as a control. Samples were also taken for SDS-PAGE analysis and microscopic observation during the growth.

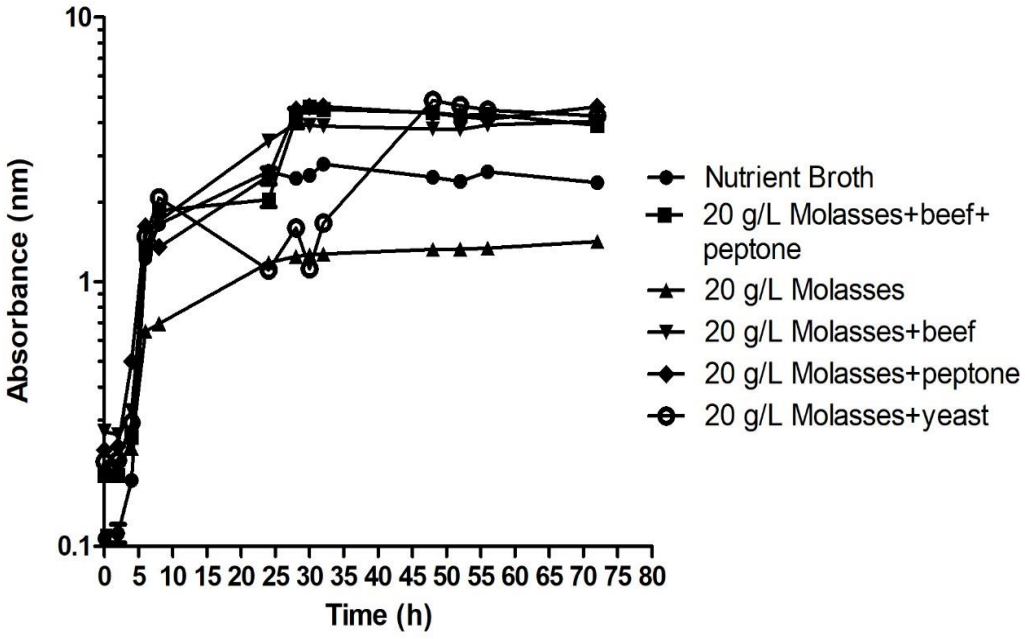


Figure 21 Growth curves of Btk-HD73 on various liquid media containing 20 g/L molasses.
Cultures were incubated at 30 ° C with 250 rpm constant shaking and the absorbance measurements were taken at 600 nm.

When the growth curve was examined (Figure 21): **(1)** growth was least in the medium with molasses only; **(2)** when beef extract, peptone or yeast extract was added to molasses, it was observed that the growth was better than that of the conventional NB.

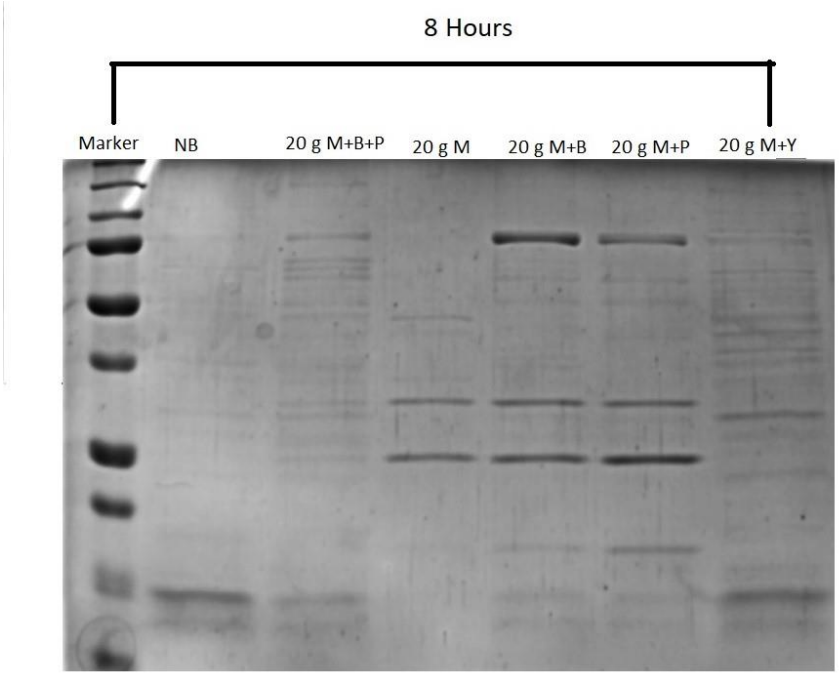


Figure 22 Btk-HD73 total protein SDS-PAGE analysis at 8th hours in different media containing 20 g / L molasses.

The change in protein profile was observed by SDS-PAGE analysis at 24th hours depending on the growth of HD-73 strain in different media containing 20 g / L molasses. The values next to the left column of the marker (M) are given in kDa. M: molasses; B: beef extract; P: peptone; Y: yeast extract.

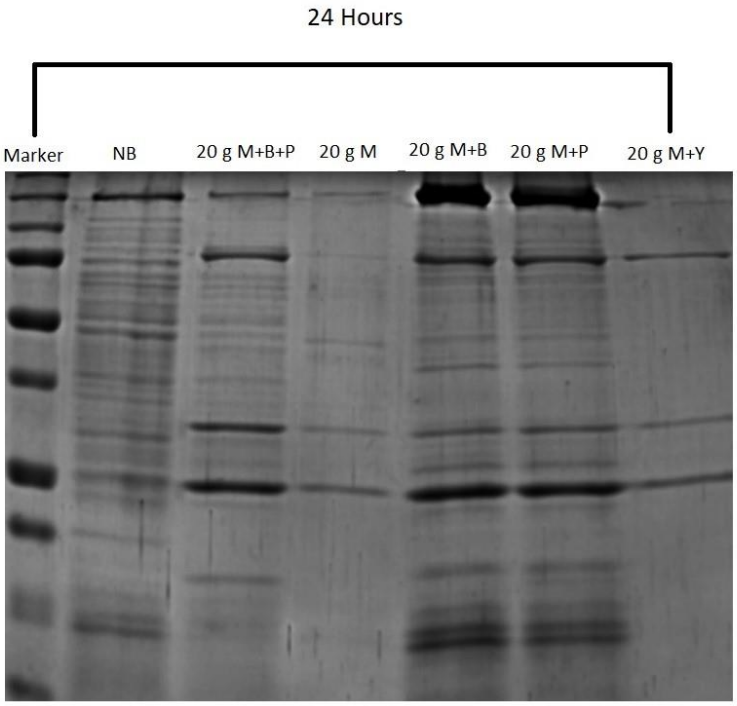


Figure 23 Btk-HD73 total protein SDS-PAGE analysis at 24th hours in different media containing 20 g / L molasses.

The change in protein profile was observed by SDS-PAGE analysis at 24th hours depending on the growth of HD-73 strain in different media containing 20 g / L molasses. The values next to the left column of the marker (M) are given in kDa. M: molasses; B: beef extract; P: peptone; Y: yeast extract.

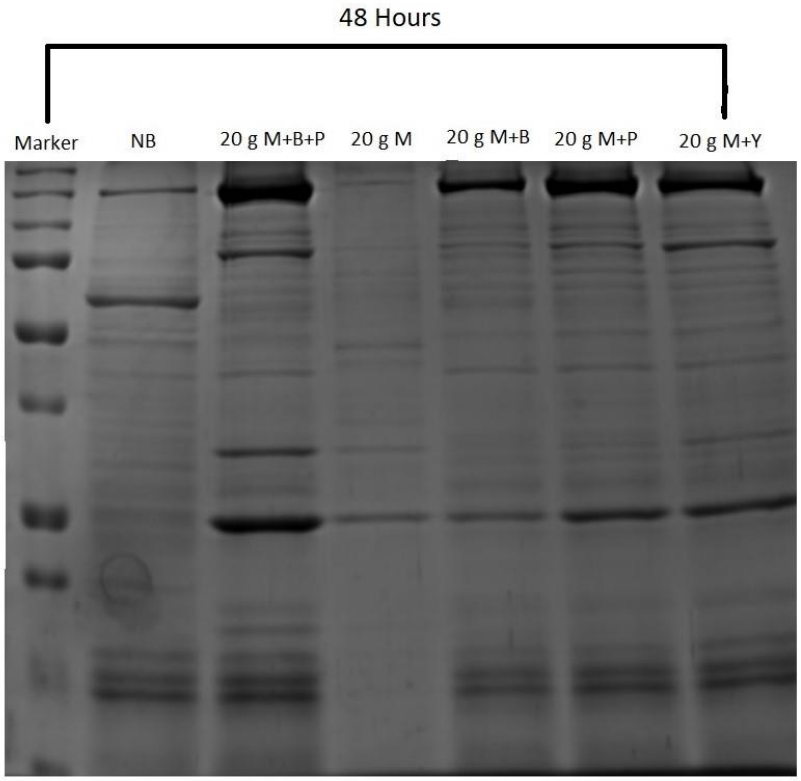


Figure 24 Btk-HD73 total protein SDS-PAGE analysis at 48th hours in different media containing 20 g / L molasses.

The change in protein profile was observed by SDS-PAGE analysis at 48th hours depending on the growth of HD-73 strain in different media containing 20 g / L molasses. The values next to the left column of the marker (M) are given in kDa. M: molasses; B: beef extract; P: peptone; Y: yeast extract.

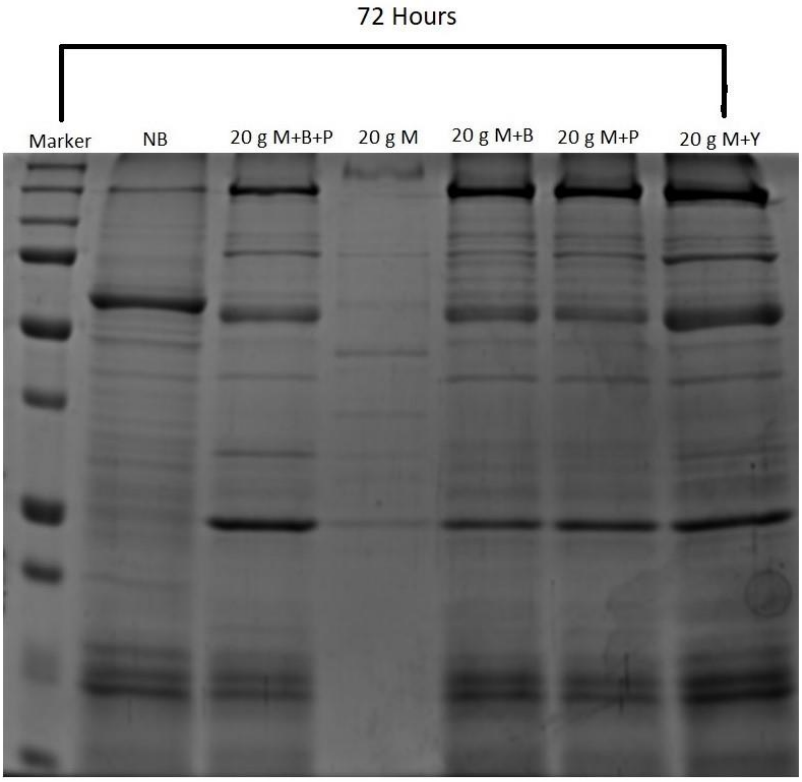


Figure 25 Btk-HD73 total protein SDS-PAGE analysis at 72th hours in different media containing 20 g / L molasses.

The change in protein profile was observed by SDS-PAGE analysis at 72th hours depending on the growth of HD-73 strain in different media containing 20 g / L molasses. The values next to the left column of the marker (M) are given in kDa. M: molasses; B: beef extract; P: peptone; Y: yeast extract.

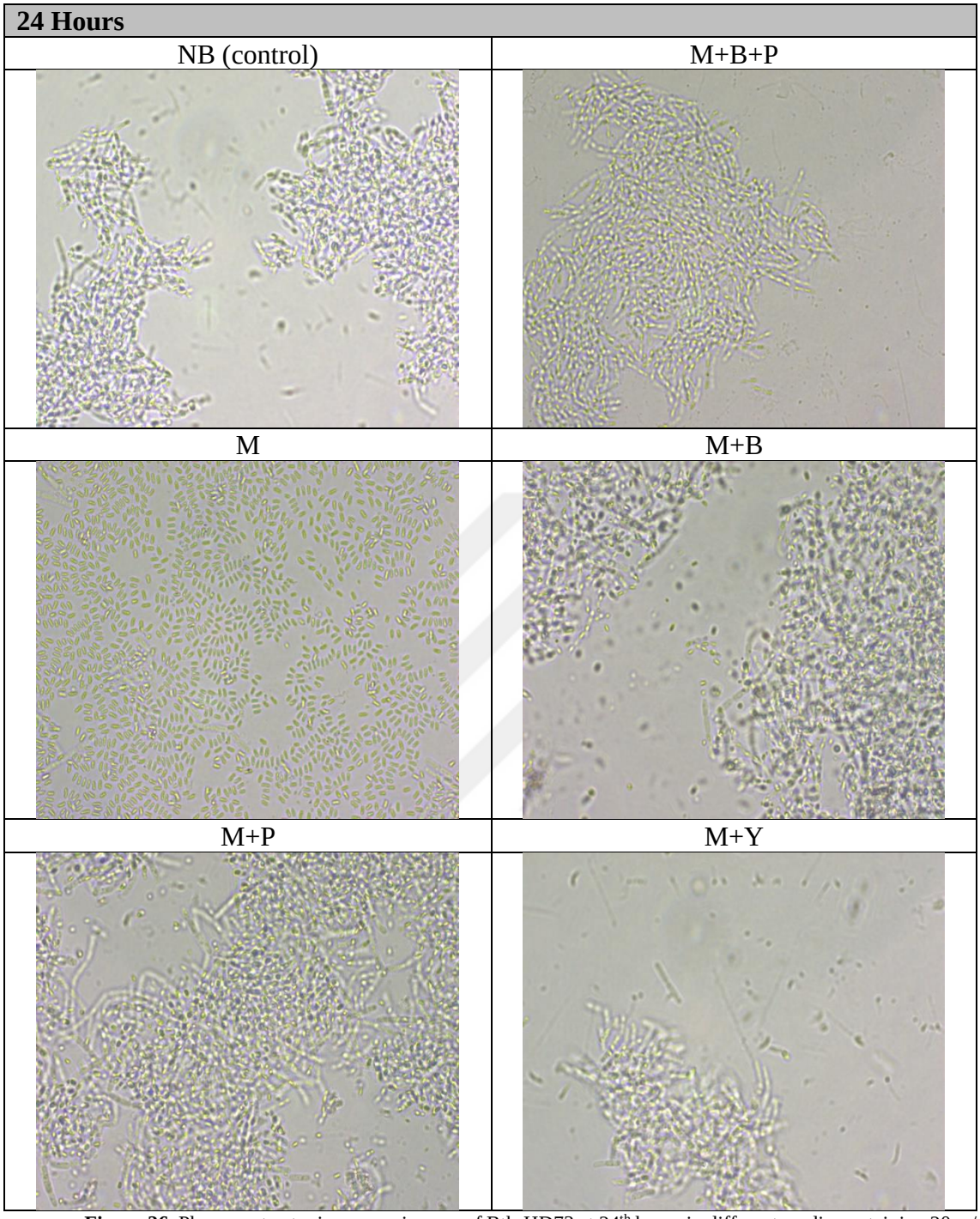


Figure 26 Phase contrast microscope images of Btk-HD73 at 24th hours in different media containing 20 g / L of molasses

Culture was incubated in liquid media containing molasses with varying concentrations at 30 ℃ with 250 rpm shaking for 72 hours . Samples were examined at 100X magnification under a phase contrast microscope at 24th hour. M: molasses; B: beef extract; P: peptone; Y: yeast extract.

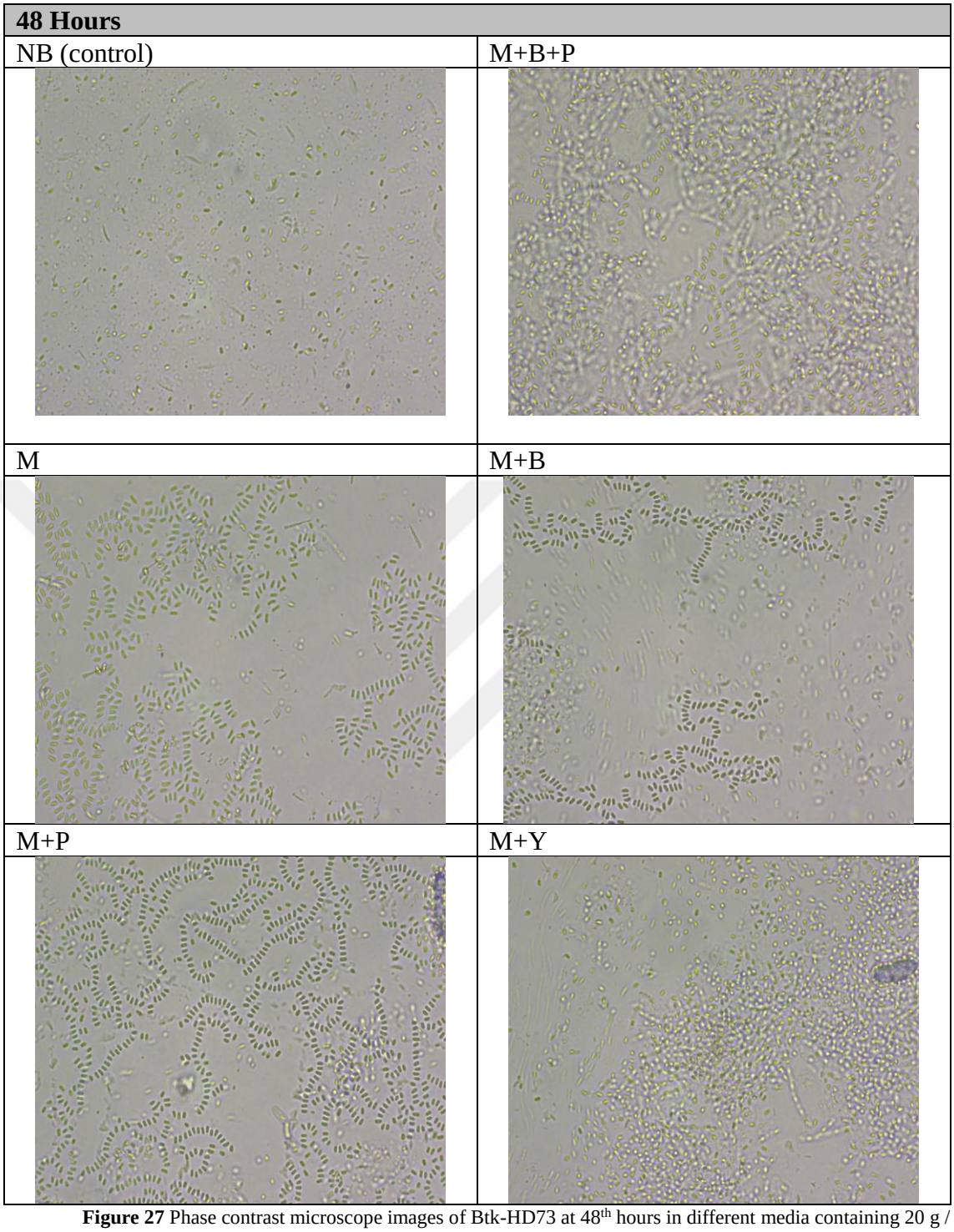


Figure 27 Phase contrast microscope images of Btk-HD73 at 48th hours in different media containing 20 g / L of molasses.

Culture was incubated in liquid media containing molasses with varying concentrations at 30 ℃ with 250 rpm shaking for 72 hours. Samples were examined at 100X magnification under a phase contrast microscope at 48th hour. M: molasses; B: beef extract; P: peptone; Y: yeast extract.

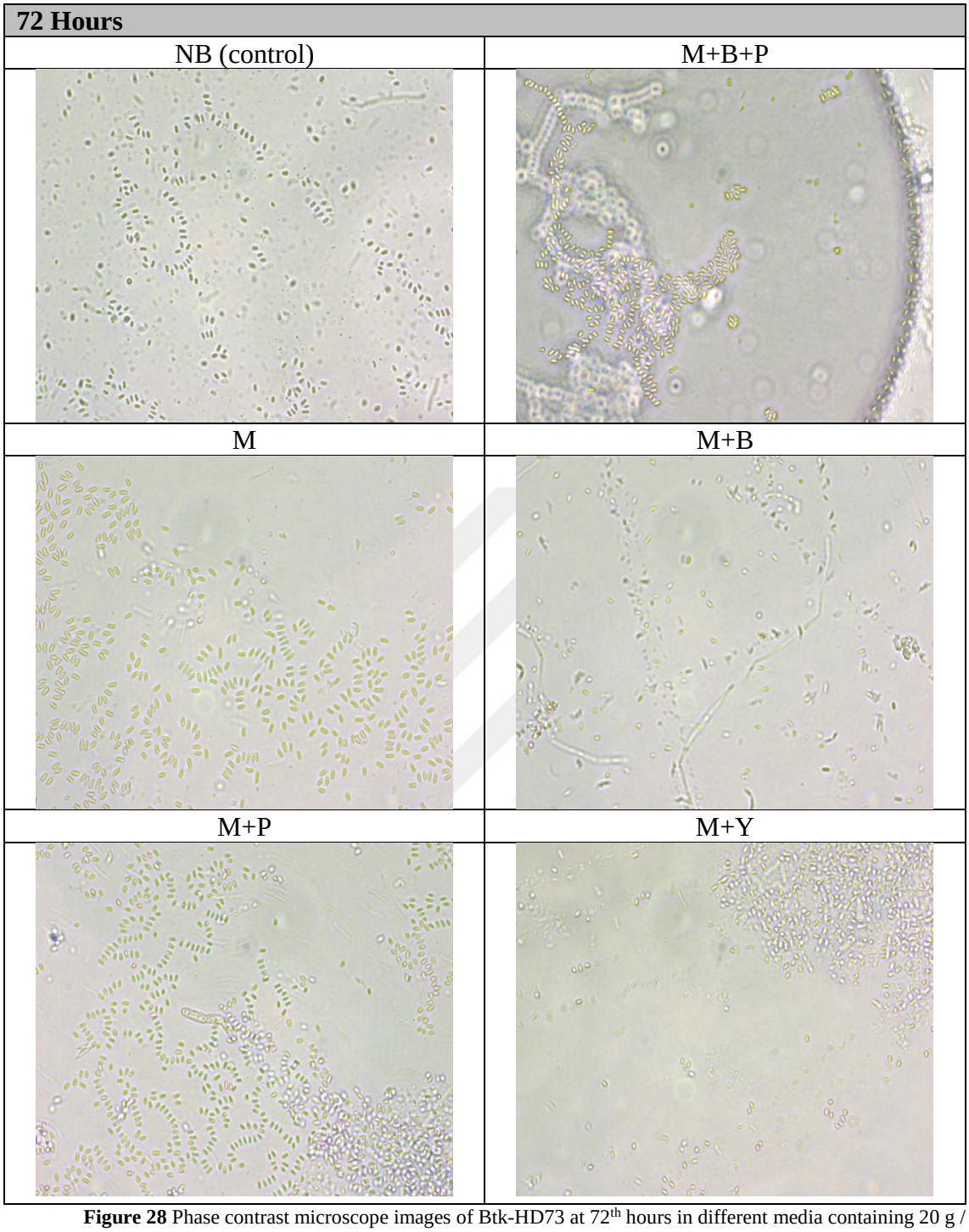


Figure 28 Phase contrast microscope images of Btk-HD73 at 72th hours in different media containing 20 g / L of molasses.

Culture was incubated in liquid media containing molasses with varying concentrations at 30℃ with 250 rpm shaking for 72 hours. Samples were examined at 100X magnification under a phase contrast microscope at 72th hour. M: molasses; B: beef extract; P: peptone; Y: yeast extract.

When the microscope images, growth curves and the results of SDS-PAGE analysis were evaluated together: **(1)** as seen in SDS-PAGE analyses addition of either peptone or beef extract caused an increase in toxin production during the first 24 hours of incubation; **(2)** toxin production in M+B+P and M+Y was comparable to that of M+P and M+B only after 48 hours of incubation; **(3)** at 72nd hour in all media conditions toxin production was almost the same except for M and NB; **(4)** Btk-HD73 sporulation was highest in medium containing molasses only, however toxin production was lowest and almost undetectable in this medium. This showed that at least under these growth conditions there was not a lineer relationship between sporulation and toxin production for Btk-HD73.

Then we wanted to compare sporulation and toxin production of Btk-HD73 both in MP medium and NYSM, which is a widely used sporulation medium for *Bacillus* species. To this end we obtained growth curves (Figure 29) and checked for toxin production (Figure 30 and 31) and sporulation (Figure 32, 33 and 34).

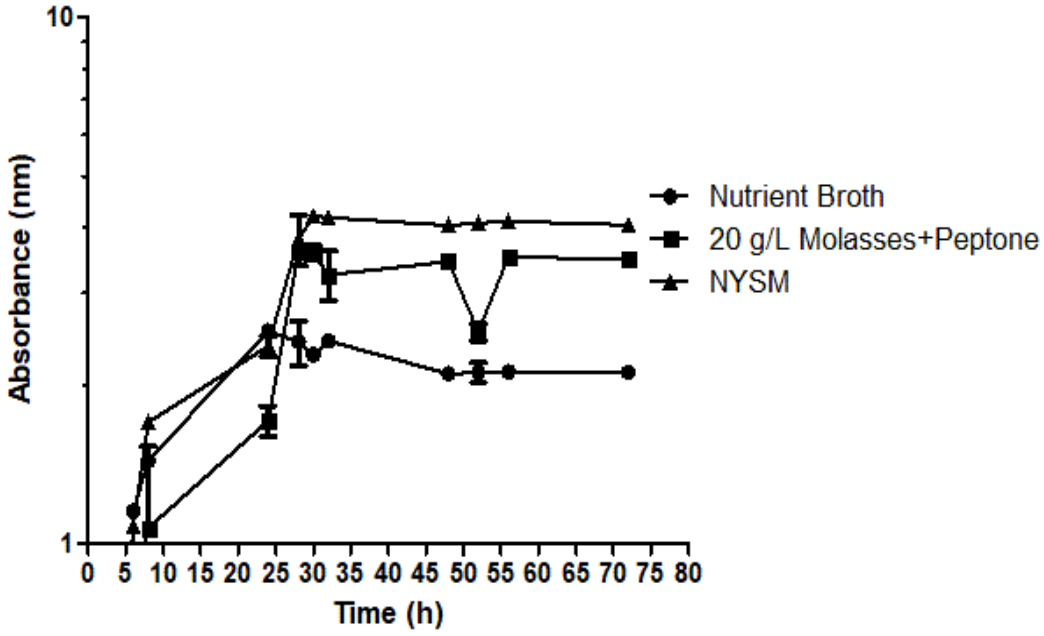


Figure 29 Growth curves of Btk-HD73 in MPM, NB and NYSM.
Cultures were incubated at 30 ° C with 250 rpm constant shaking and the absorbance measurements were taken at 600 nm. MPM: 20 g / L Molasses + peptone; NB: Nutrient broth; NYSM: beef extract + peptone + yeast extract + salt content).

When the growth curve was examined: **(1)** bacterial growth was higher in molasses + peptone compared to NB; **(2)** bacterial growth was highest in NYSM medium compared to other media tested under these conditions.

Samples were also collected from these media during the growth at various time points to check toxin production by SDS-PAGE analysis (Figure 30 and 31) and sporulation via microscopic analysis (Figure 32, 33 and 34).

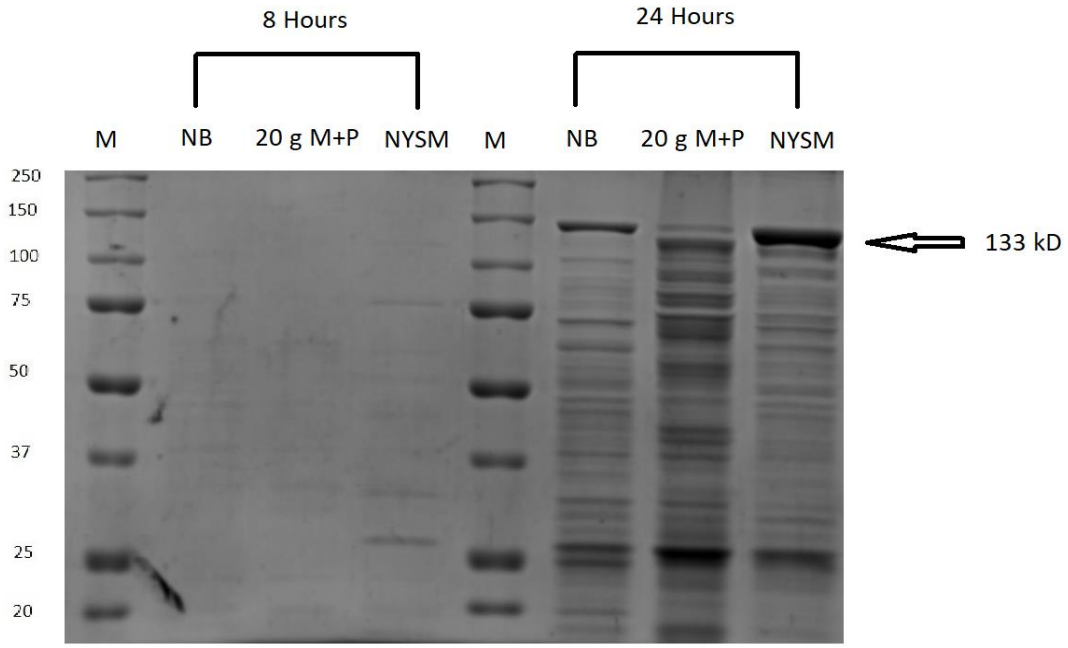


Figure 30 Btk-HD73 total protein SDS-PAGE analysis at 8th and 24th hours in MPM, Nutrient broth and NYSM.

The change in protein profile was observed by SDS-PAGE analysis at 8th and 24th hours depending on the growth of HD-73 strain 20 g / L Molasses + peptone, Nutrient broth and NYSM (beef extract + peptone + yeast extract + salt content). The values next to the left column of the marker (M) are given in kDa. M: molasses; P: peptone

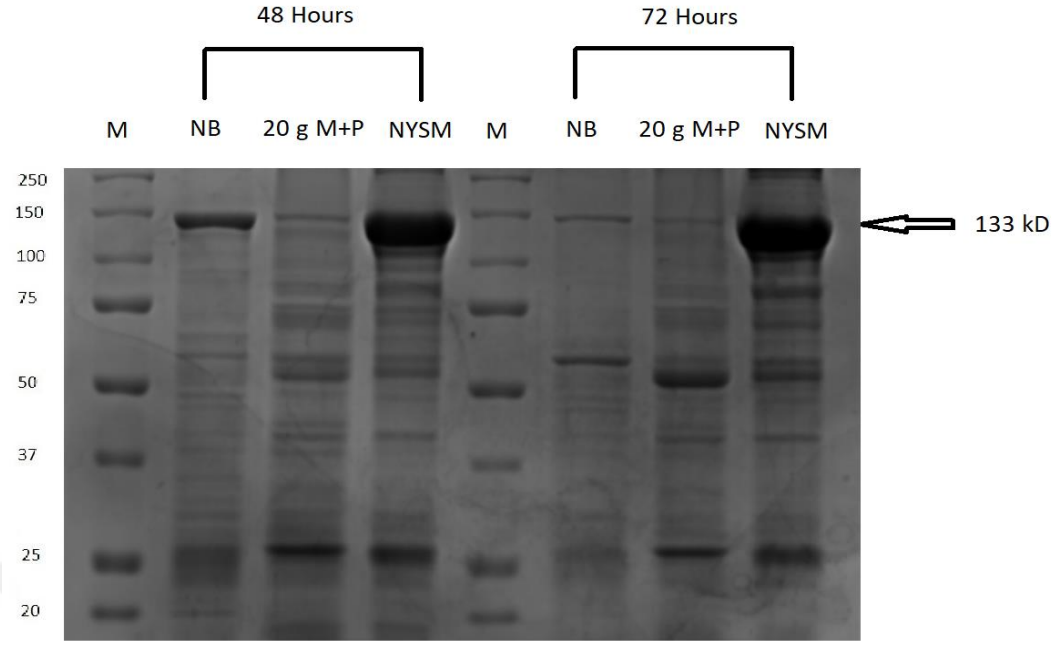


Figure 31 Btk-HD73 total protein SDS-PAGE analysis at 48th and 72th hours in MPM, Nutrient broth and NYSM.

The change in protein profile was observed by SDS-PAGE analysis at 48th and 72th depending on the growth of HD-73 strain 20 g / L Molasses + peptone, Nutrient broth and NYSM (beef extract + peptone + yeast extract + salt content). The values next to the left column of the marker (M) are given in kDa. M: molasses; P: peptone

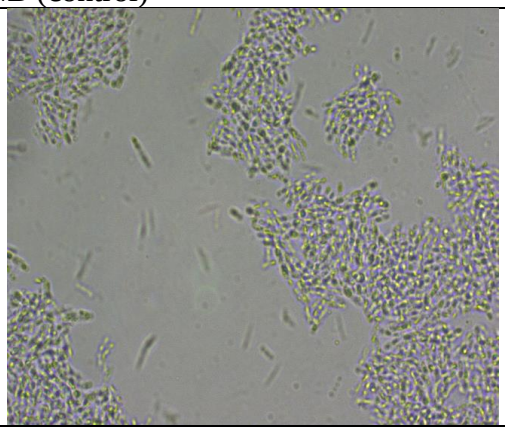
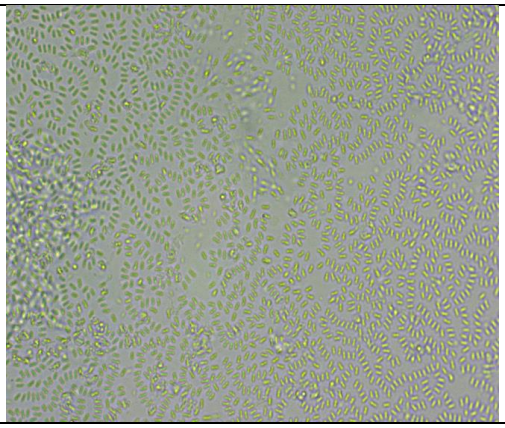
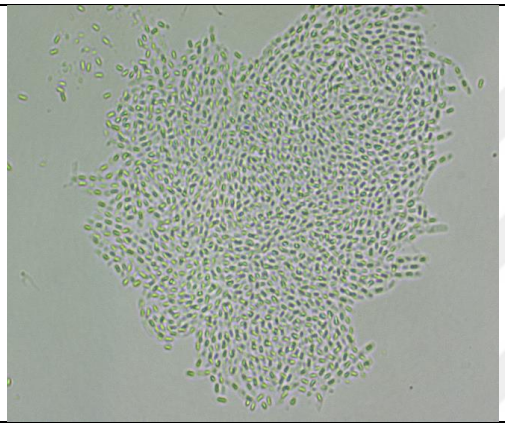
24 Hours	
NB (control)	M+P
	
NYSM	
	

Figure 32 Phase contrast microscope images of Btk-HD73 at 24th hours in MPM, Nutrient broth and NYSM. Culture was incubated in 20 g / L Molasses + peptone, Nutrient broth and NYSM (beef extract + peptone + yeast extract + salt content) at 30℃ with 250 rpm shaking for 72 hours. Samples were examined at 100X magnification under a phase contrast microscope at 24th hour. M: molasses; P: peptone

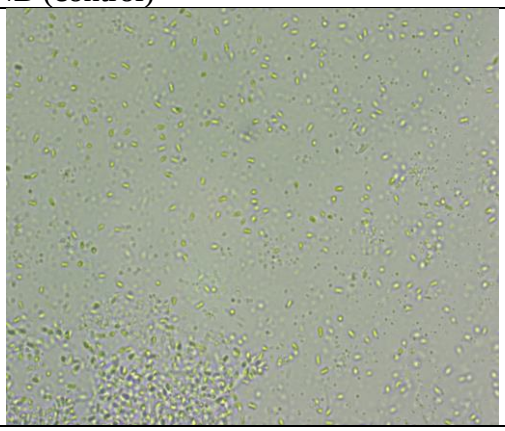
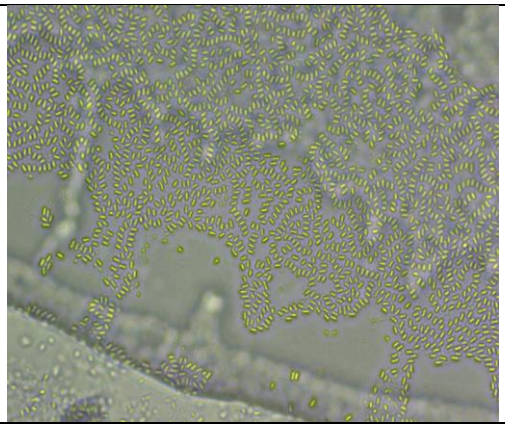
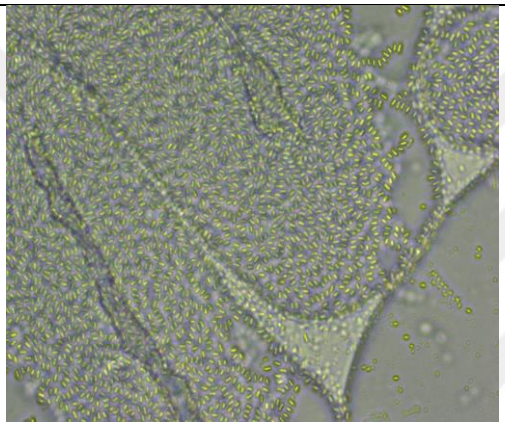
48 Hours	
NB (control)	M+P
	
NYSM	
	

Figure 33 Phase contrast microscope images of Btk-HD73 at 48th hours in MPM, Nutrient broth and NYSM. Culture was incubated in 20 g / L Molasses + peptone, Nutrient broth and NYSM (beef extract + peptone + yeast extract + salt content) at 30℃ with 250 rpm shaking for 72 hours. Samples were examined at 100X magnification under a phase contrast microscope at 48th hour. M: molasses; P: peptone

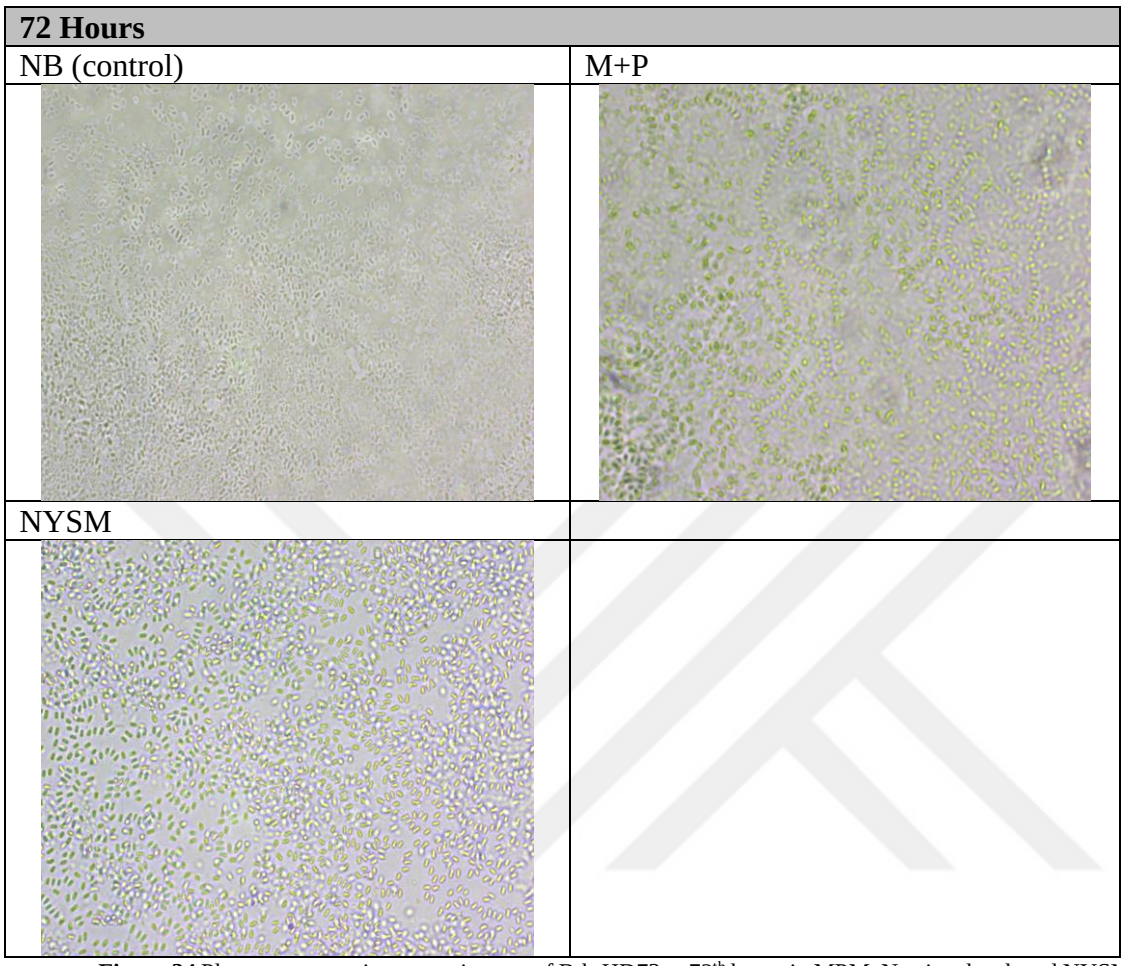


Figure 34 Phase contrast microscope images of Btk-HD73 at 72th hours in MPM, Nutrient broth and NYSM. Culture was incubated in 20 g / L Molasses + peptone, Nutrient Broth and NYSM (beef extract + peptone + yeast extract + salt content) at 30℃ with 250 rpm shaking for 72 hours. Samples were examined at 100X magnification under a phase contrast microscope at 72 hours. M: molasses; P: peptone

When the obtained microscope images, growth curves and SDS-PAGE results were evaluated together: **(1)** it was observed that both growth and toxin production was highest in NYSM for all time points; **(2)** toxin production in NYSM was observed starting from 24th hour sample and was highest at 48th and 72nd hour samples; **(3)** sporulation was observable in samples collected starting from the first 24 hours and increased in later samples. Although sporulation at 48th and 72nd hour samples of M+P and NYSM was similar, toxin production could not be detected in M+P samples. This again showed that there is no positive correlation between sporulation and toxin production for Btk-HD73 in media containing molasses.

4.3.2. Effect of salt solution in molasses media on sporulation and toxin production

NYSM contained Ca^{2+}, Mg^{2+} and Mn^{2+} salts and these salts are associated with both the sporulation and toxin production. Therefore, we wanted to see the effect of these salts on toxin production in our media containing molasses. To this end MP, MB, MBP media and their salt solution added derivations were compared against NYSM and TBL especially for toxin production. TBL is another medium commonly used for sporulation in *Bacillus* species.

Btk-HD73 was grown in selected media and their salt added derivations at 30°C with 250 rpm constant shaking for 72 hours (Figure 35). Samples collected at every 24 hours to check toxin production using SDS-PAGE analysis (Figure 36, 37 and 38) and sporulation via microscopic analysis (Figure 39, 40 and 41).

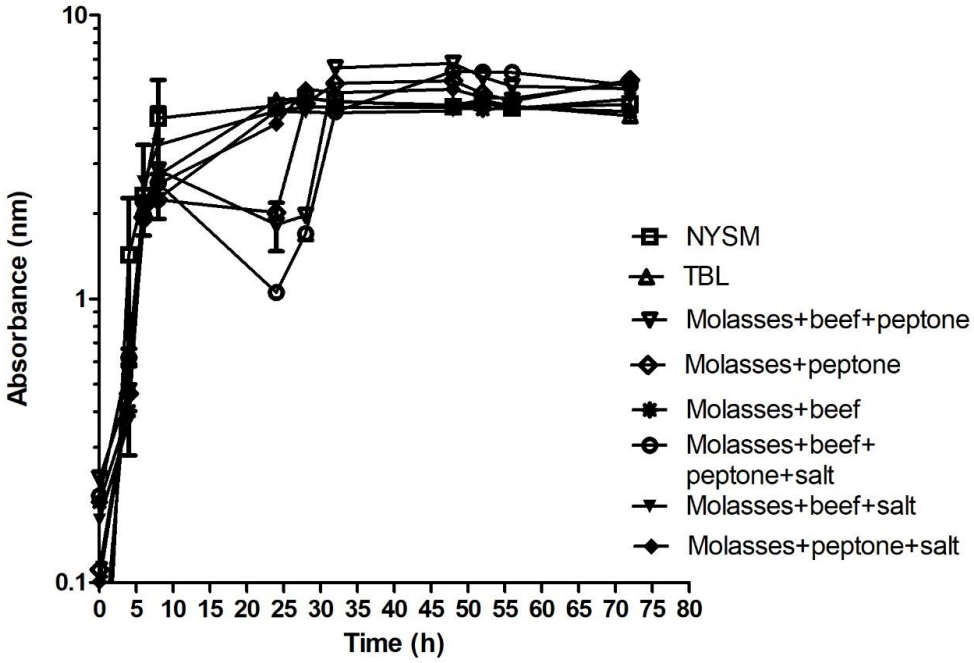


Figure 35 Growth curves of Btk-HD73 in MBPM, MPM, MBM, MBPSM, MBSM, MPSM, NYSM and TBL.

Cultures were incubated at 30 ° C with 250 rpm constant shaking and the absorbance measurements were taken at 600 nm. MBPM: 20 g / L Molasses + beef extract + peptone; MPM: 20 g / L Molasses + peptone; MBM: 20 g / L Molasses + beef extract; MBPSM: 20 g / L Molasses + beef extract + peptone+ salt contents; MBSM: 20 g / L Molasses + beef extract+ salt contents; MPSM: 20 g / L Molasses + peptone+ salt contents. Salt (Salt solution; $CaCl_2$ + $MnCl_2$ + $MgCl_2$).

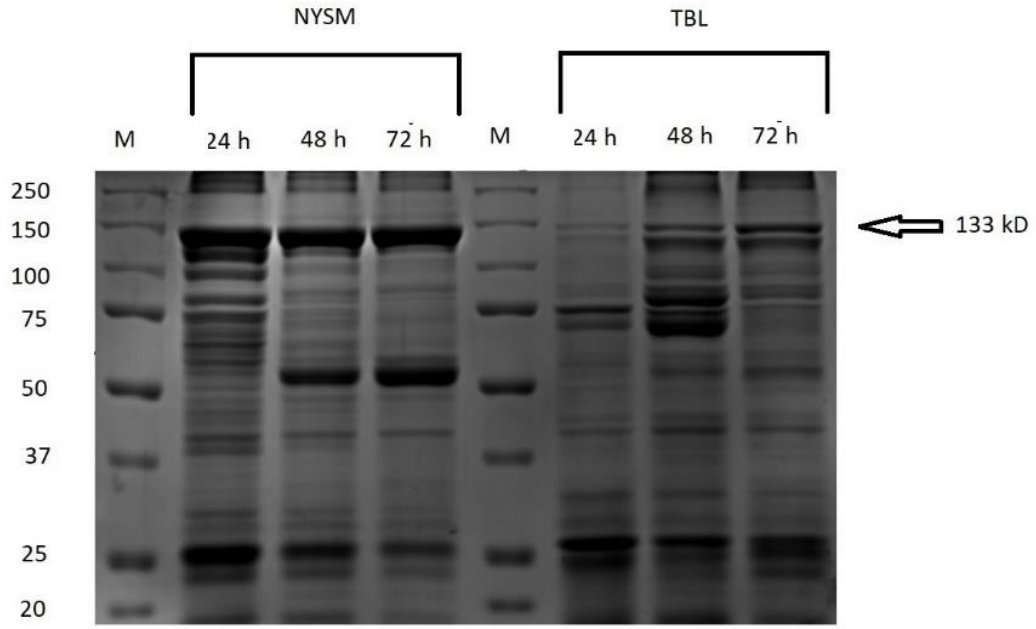


Figure 36 Btk-HD73 total protein SDS-PAGE analysis at 24th,48th and 72th hours in NYSM and TBL.

The change in protein profile was observed by SDS-PAGE analysis at 24th,48th and 72th hours depending on the growth of HD-73 strain in NYSM and TBL. The values next to the left column of the marker (M) are given in kDa.

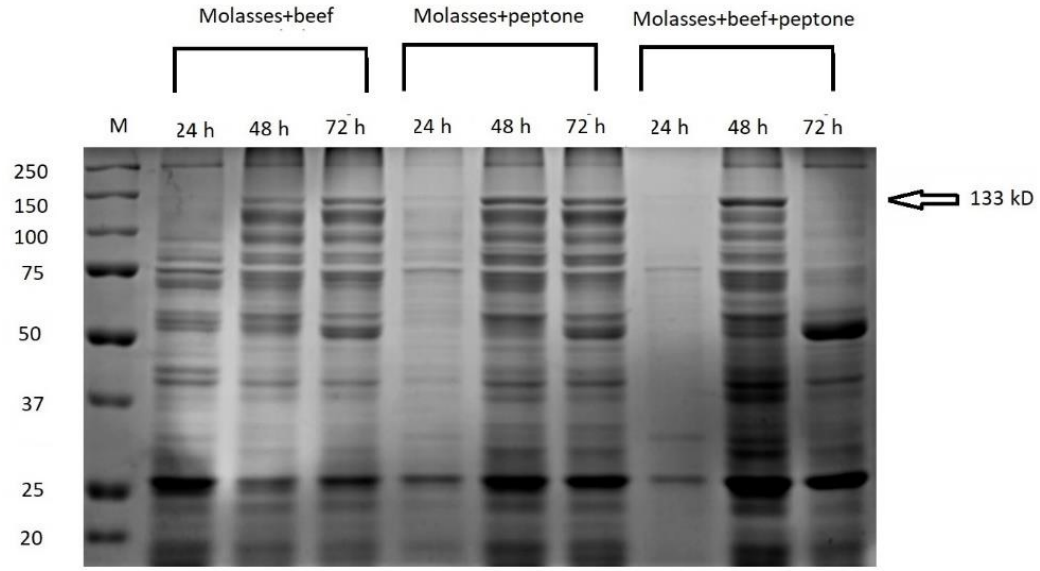


Figure 37 Btk-HD73 total protein SDS-PAGE analysis at 24th,48th and 72th hours in MBM, MPM and MBPM.

The change in protein profile was observed by SDS-PAGE analysis at 24th,48th and 72th hours depending on the growth of HD-73 strain in in MBM, MPM and MBPM. MBPM: 20 g / L Molasses + beef extract + peptone; MPM: 20 g / L Molasses + peptone; MBM: 20 g / L Molasses + beef extract. The values next to the left column of the marker (M) are given in kDa.

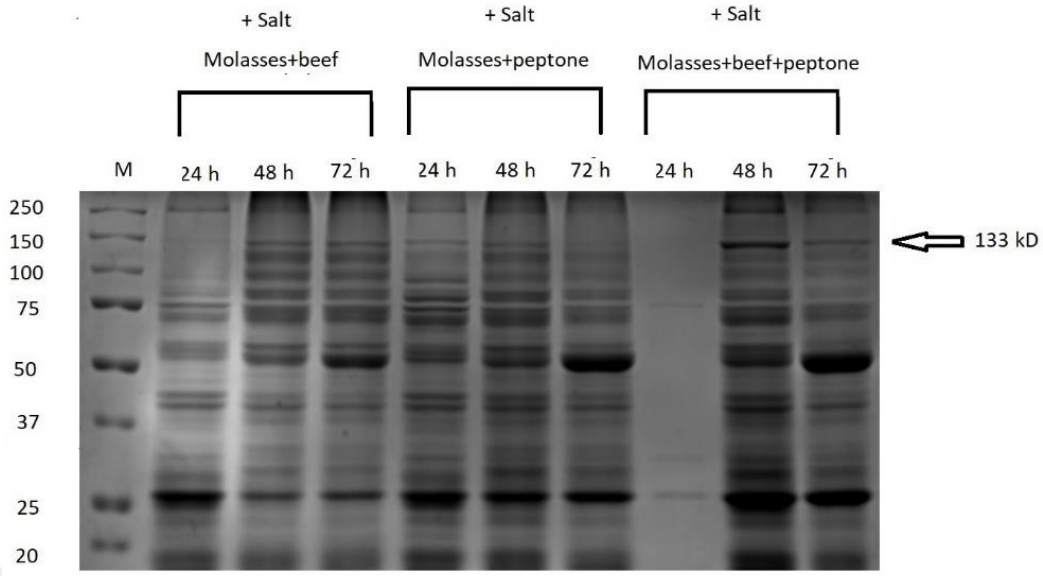


Figure 38 Btk-HD73 total protein SDS-PAGE analysis at 24th,48th and 72th hours in MBSM, MPSM and MBPSM.

The change in protein profile was observed by SDS-PAGE analysis at 24th,48th and 72th hours depending on the growth of HD-73 strain in in MBSM, MPSM and MBPSM. MBPSM: 20 g / L Molasses + beef extract + peptone+ salt contents; MBSM: 20 g / L Molasses + beef extract+ salt contents; MPSM: 20 g / L Molasses + peptone+ salt contents. Salt (Salt solution; $CaCl_2$ + $MnCl_2$ + $MgCl_2$). The values next to the left column of the marker (M) are given in kDa.

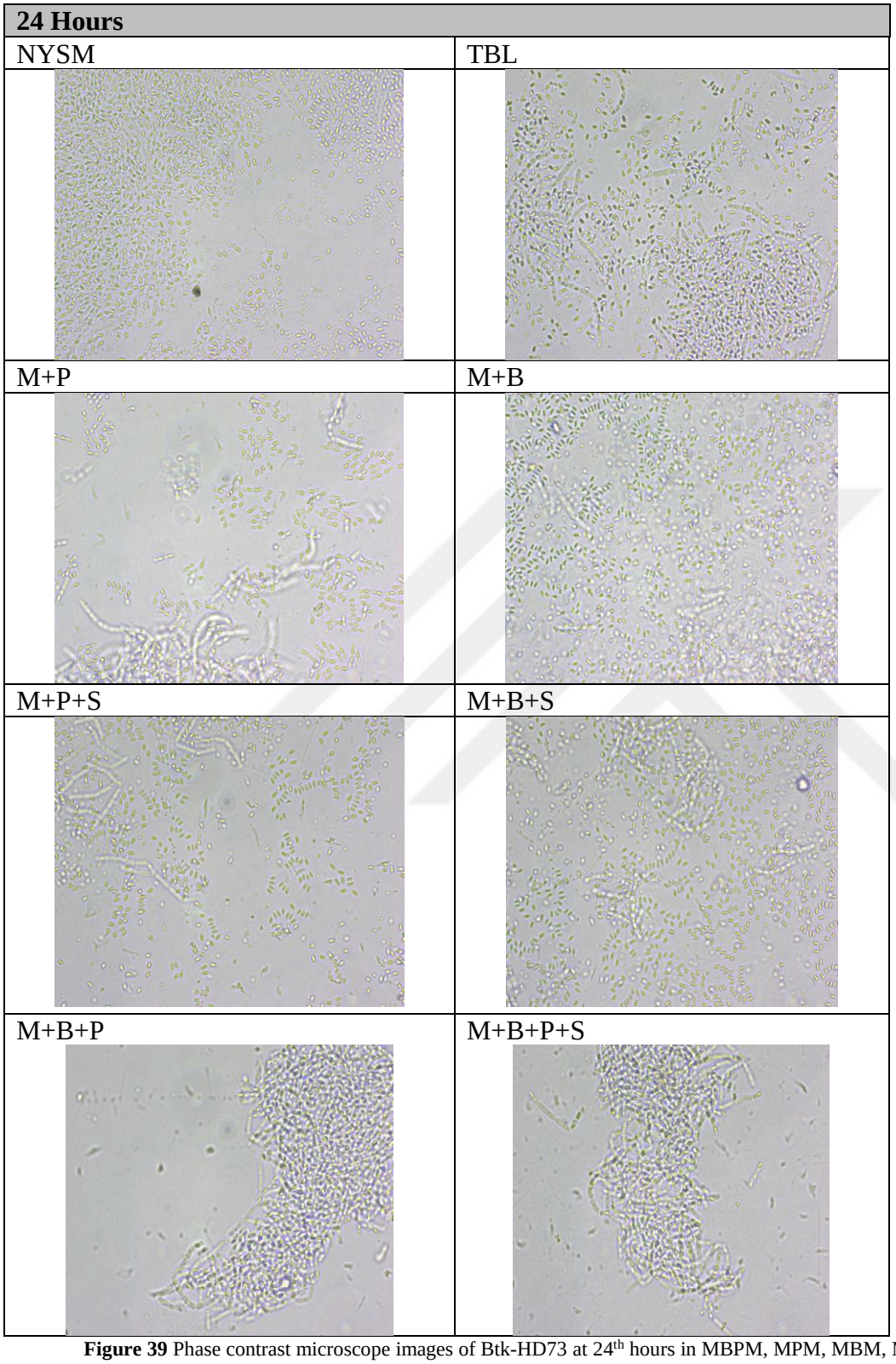


Figure 39 Phase contrast microscope images of Btk-HD73 at 24th hours in MBPM, MPM, MBM, MBPSM, MBSM, MPSM, NYSM and TBL.

Culture was incubated in MBPM, MPM, MBM, MBPSM, MBSM, MPSM, NYSM and TBL at 30℃ with 250 rpm shaking for 72 hours. Samples were examined at 100X magnification under a phase contrast microscope at 72th hour. MBPM: 20 g / L Molasses + beef extract + peptone; MPM: 20 g / L Molasses + peptone; MBM: 20 g / L Molasses + beef extract; MBPSM: 20 g / L Molasses + beef extract + peptone+ salt contents; MBSM: 20 g / L Molasses + beef extract+ salt contents; MPSM: 20 g / L Molasses + peptone+ salt contents. Salt (Salt solution; $CaCl_2$ + $MnCl_2$ + $MgCl_2$).

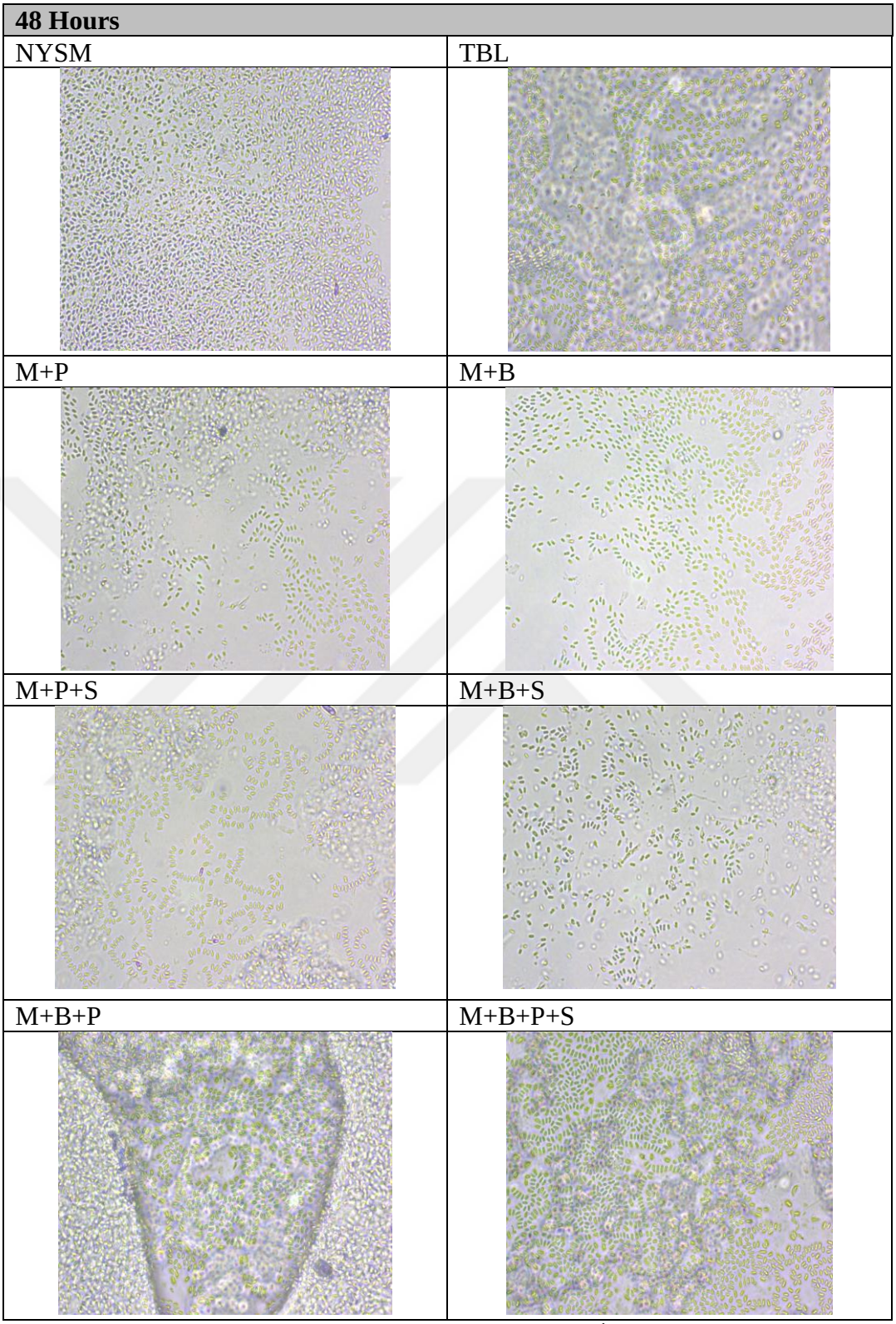


Figure 40 Phase contrast microscope images of Btk-HD73 at 48th hours in MBPM, MPM, MBM, MBPSM, MBSM, MPSM, NYSM and TBL.

Culture was incubated in MBPM, MPM, MBM, MBPSM, MBSM, MPSM, NYSM and TBL at 30℃ with 250 rpm shaking for 72 hours. Samples were examined at 100X magnification under a phase contrast microscope at 72th hour. MBPM: 20 g / L Molasses + beef extract + peptone; MPM: 20 g / L Molasses + peptone; MBM: 20 g / L Molasses + beef extract; MBPSM: 20 g / L Molasses + beef extract + peptone + salt contents; MBSM: 20 g / L Molasses + beef extract+ salt contents; MPSM: 20 g / L Molasses + peptone+ salt contents. Salt (Salt solution; $CaCl_2$ + $MnCl_2$ + $MgCl_2$).

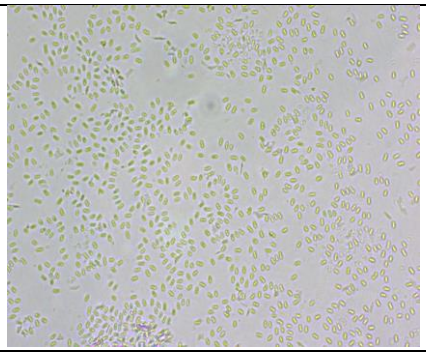
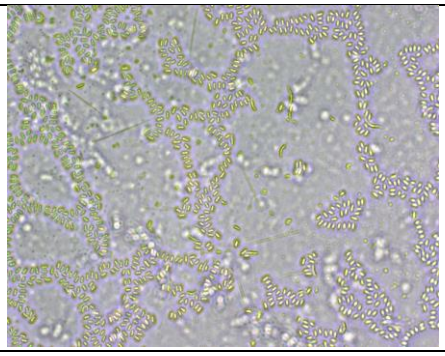
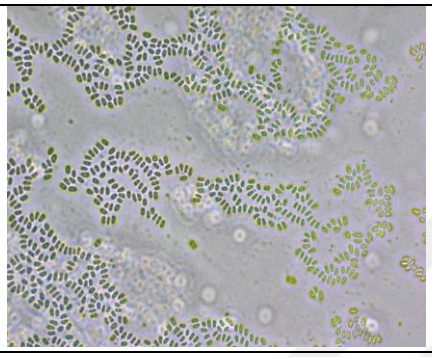
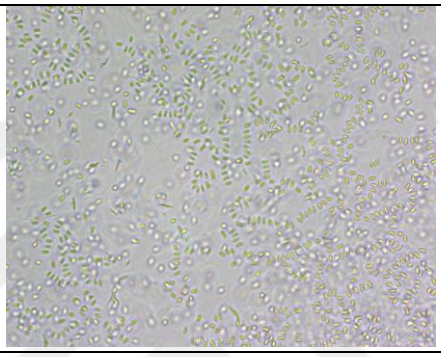
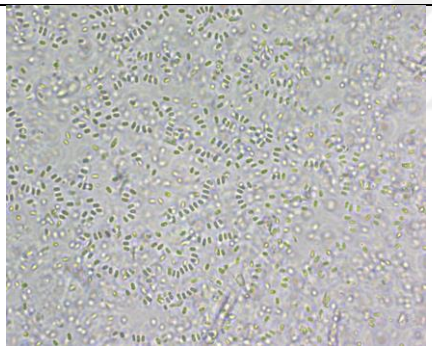
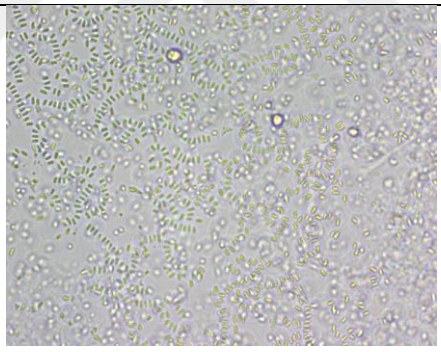
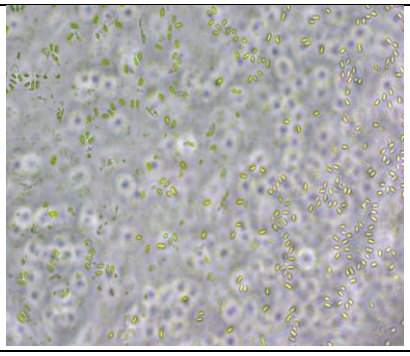
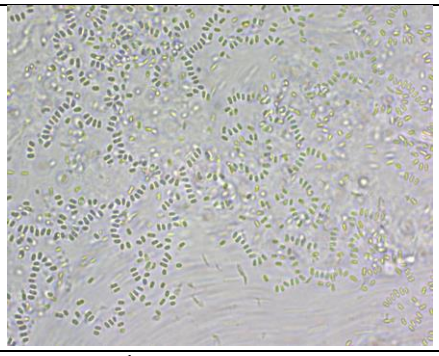
72 Hours	
NYSM	TBL
	
M+P	M+B
	
M+P+S	M+B+S
	
M+B+P	M+B+P+S
	

Figure 41 Phase contrast microscope images of Btk-HD73 at 72th hours in MBPM, MPM, MBM, MBPSM, MBSM, MPSM, NYSM and TBL.

Culture was incubated in MBPM, MPM, MBM, MBPSM, MBSM, MPSM, NYSM and TBL at 30℃ with 250 rpm shaking for 72 hours. Samples were examined at 100X magnification under a phase contrast microscope at 72th hour. MBPM: 20 g / L Molasses + beef extract + peptone; MPM: 20 g / L Molasses + peptone; MBM: 20 g / L Molasses + beef extract; MBPSM: 20 g / L Molasses + beef extract + peptone + salt contents; MBSM: 20 g / L Molasses + beef extract+ salt contents; MPSM: 20 g / L Molasses + peptone+ salt contents. Salt (Salt solution; $CaCl_2$ + $MnCl_2$ + $MgCl_2$).

When these results were evaluated: **(1)** the addition of salt did not affect the growth rate of Btk-HD73 in these medium varieties; **(2)** the use of beef extract, peptone or both as a nitrogen source did not make any positive contribution to the toxin production or growth compared to NYSM; **(3)** addition of salt to molasses medium derivatives did not cause an increase in toxin production compared to no salt added forms; **(4)** spore numbers were comparable in all media tested, again indicating that at least in molasses containing media Btk-HD73 sporulation is not positively correlated with toxin production; **(5)** highest toxin production was obtained in NYSM compared to both salt added molasses medium derivatives and TBL.

We obtained highest toxin production in NYSM and therefore, we focused next contents of this medium. To this end, growth (Figure 42), toxin production (Figure 43 and 44) and sporulation (Figure 45, 46, 47, 48, 49 and 50), 24, 48, 72 hours' images) were tested in NYSM, NYM (NYSM without salt), MYSM and MYM (MYSM without salt). NYM was containing all NYSM contents (beef extract, yeast extract, peptone and salt solution) except the salt solution. MYM was containing all MYSM contents (molasses, yeast extract and salt solution) except the salt solution.

First Btk-HD73 was grown in these selected media at 30°C with 250 rpm constant shaking for 72 hours (Figure 42).

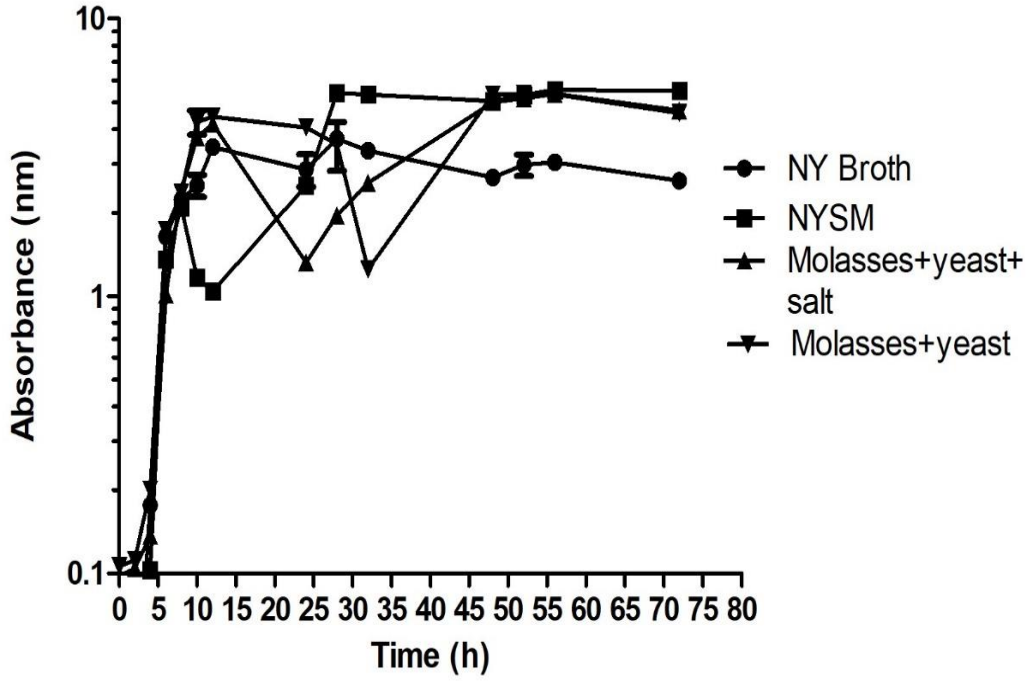


Figure 42 Growth curves of Btk-HD73 in MYSM, MYM and NYSM.
Cultures were incubated at 30°C with 250 rpm constant shaking for 72 hours and the absorbance measurements were taken at 600 nm. NY (beef extract + peptone + yeast extract); NYSM (beef extract + peptone + yeast extract + salt content); MYSM: 20 g / L Molasses + yeast extract+ salt content; MYM: 20 g / L Molasses + yeast extract. Salt (Salt solution; $CaCl_2$ + $MnCl_2$ + $MgCl_2$).

When growth curves were examined, we have seen that growth of Btk-HD73 was similar in all media and also, we have noted that salt addition did not make any difference.

During growth samples were also taken at 12^{th}, 24^{th}, 48^{th} and 72^{nd} hour time points to check toxin production and sporulation.

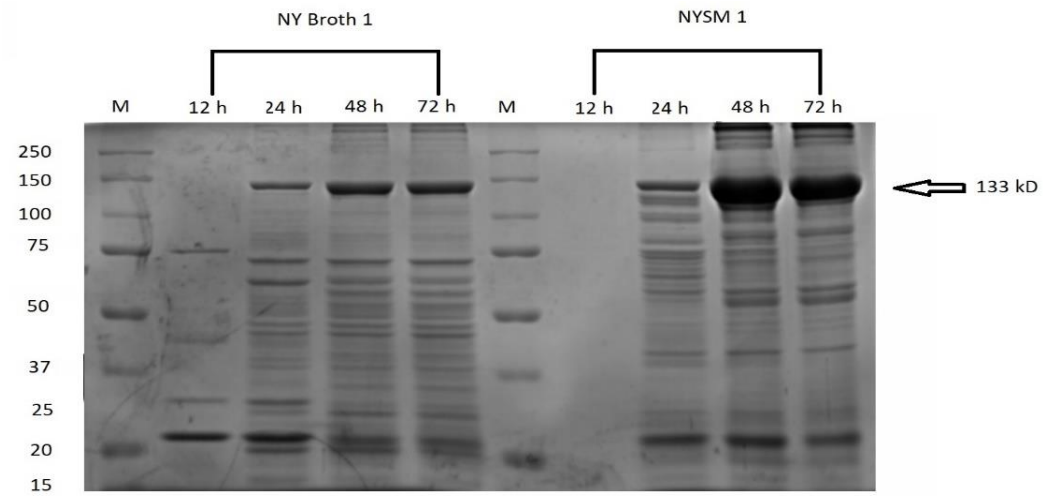


Figure 43 Btk-HD73 total protein SDS-PAGE analysis at 12th, 24th,48th and 72th hours in NY and NYSM.

The change in protein profile was observed by SDS-PAGE analysis at 12th, 24th,48th and 72th hours depending on the growth of HD-73 strain in NY and NYSM. NY (beef extract + peptone + yeast extract); NYSM (beef extract + peptone + yeast extract + salt content). Marker (M) next to the left column; values are given in kDa.

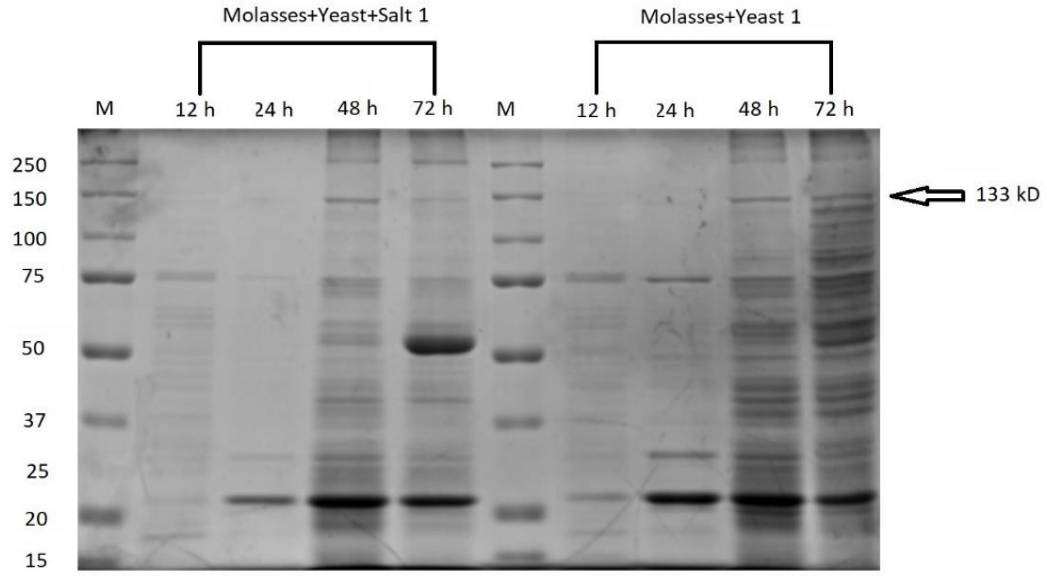


Figure 44 Btk-HD73 total protein SDS-PAGE analysis at 12th, 24th,48th and 72th hours in MYSM and MYM.

The change in protein profile was observed by SDS-PAGE analysis at 12th, 24th,48th and 72th hours depending on the growth of HD-73 strain in different media containing 20 g / L Molasses, yeast and salt solution. MYSM: 20 g / L Molasses + yeast extract+ salt content; MYM: 20 g / L Molasses + yeast extract. Salt (Salt solution; $CaCl_2$ + $MnCl_2$ + $MgCl_2$). Marker (M) next to the left column; values are given in kDa.

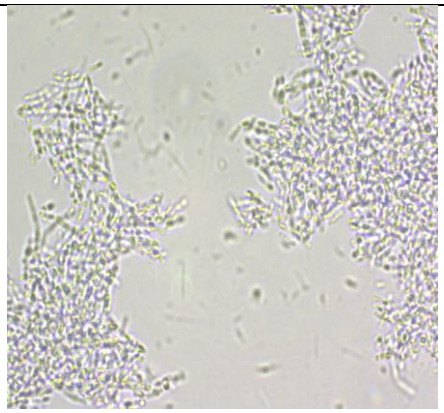
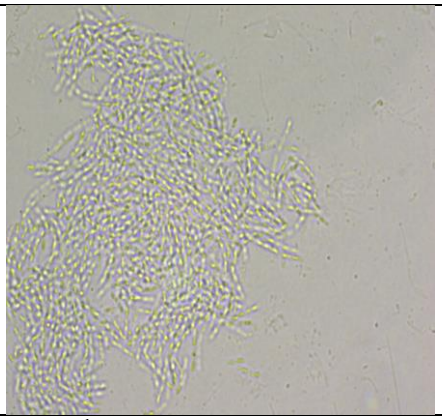
24 Hours	
NY Broth	NYSM
	

Figure 45 Phase contrast microscope images of Btk-HD73 at 24th hours in NY and NYSM.
Culture was incubated in NY and NYSM at 30℃ with 250 rpm shaking for 72 hours. Samples were examined at 100X magnification under a phase contrast microscope at 24th hour

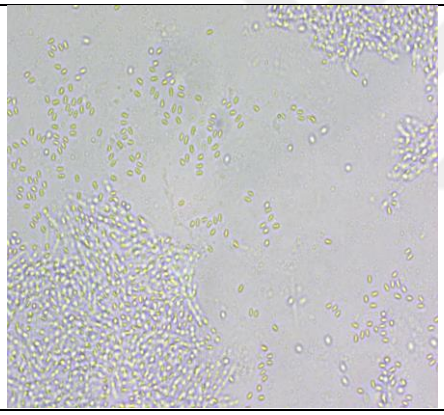
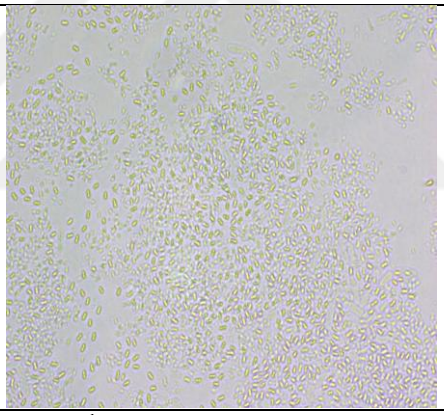
48 Hours	
NY Broth	NYSM
	

Figure 46 Phase contrast microscope images of Btk-HD73 at 48th hours in NY and NYSM.
Culture was incubated in NY and NYSM at 30℃ with 250 rpm shaking for 72 hours. Samples were examined at 100X magnification under a phase contrast microscope at 48th hour.

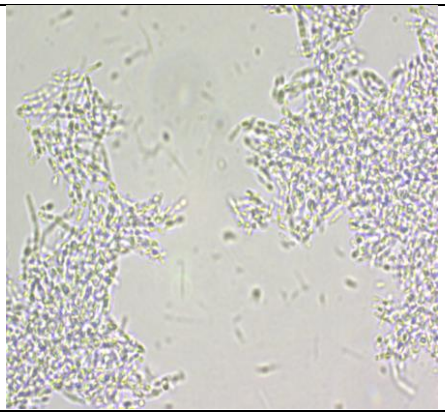
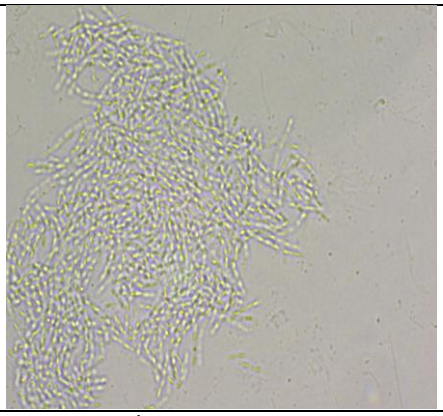
72 Hours	
NY Broth	NYSM
	

Figure 47 Phase contrast microscope images of Btk-HD73 at 72th hours in NY and NYSM.

Culture was incubated in NY and NYSM at 30℃ with 250 rpm shaking for 72 hours. Samples were examined at 100X magnification under a phase contrast microscope at 72th hour.

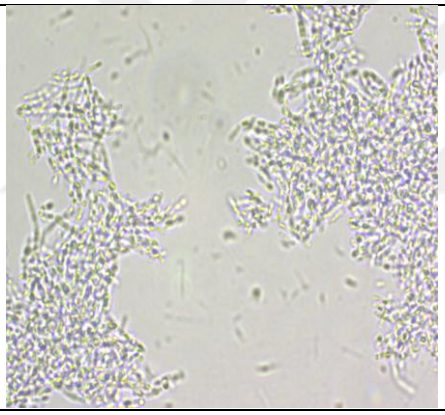
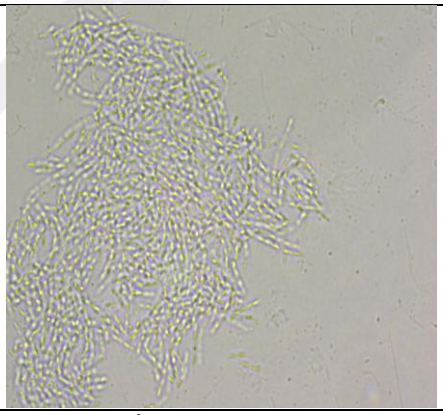
24 Hours	
M+Y	M+Y+S
	

Figure 48 Phase contrast microscope images of Btk-HD73 at 24th hours in MYM and MYSM.

Culture was incubated in MYM and MYSM at 30℃ with 250 rpm shaking for 72 hours. Samples were examined at 100X magnification under a phase contrast microscope at 24th hour. MYSM: 20 g / L Molasses + yeast extract+ salt content; MYM: 20 g / L Molasses + yeast extract. S: (Salt solution; $CaCl_2$ + $MnCl_2$ + $MgCl_2$).

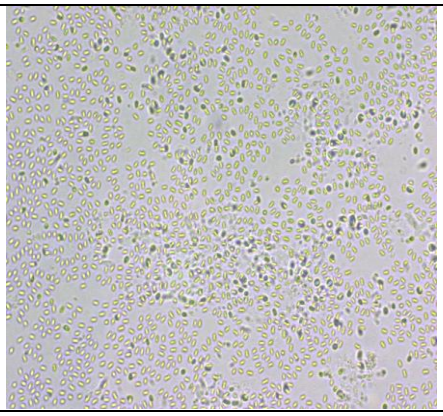
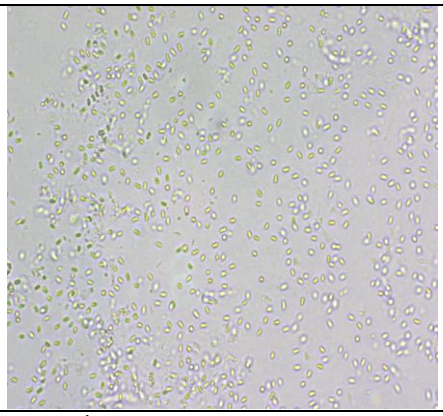
48 Hours	
M+Y	M+Y+S
	

Figure 49 Phase contrast microscope images of Btk-HD73 at 48th hours in MYM and MYSM.
Culture was incubated in MYM and MYSM at 30℃ with 250 rpm shaking for 72 hours. Samples were examined at 100X magnification under a phase contrast microscope at 48th hour. MYSM: 20 g / L Molasses + yeast extract+ salt content; MYM: 20 g / L Molasses + yeast extract. S: (Salt solution; $CaCl_2$ + $MnCl_2$ + $MgCl_2$).

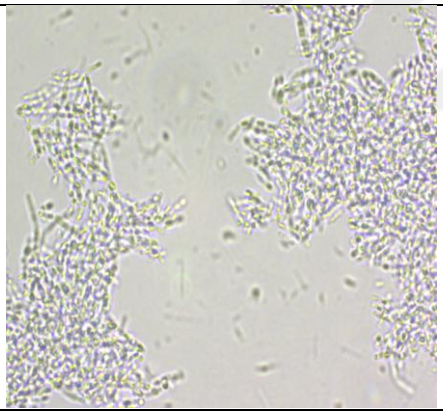
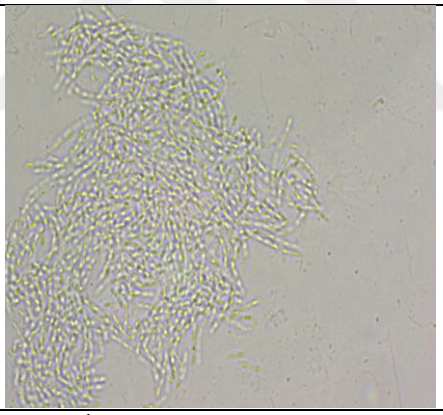
72 Hours	
M+Y	M+Y+S
	

Figure 50 Phase contrast microscope images of Btk-HD73 at 72th hours in MYM and MYSM.
Culture was incubated in MYM and MYSM at 30℃ with 250 rpm shaking for 72 hours. Samples were examined at 100X magnification under a phase contrast microscope at 24th hour. MYSM: 20 g / L Molasses + yeast extract+ salt content; MYM: 20 g / L Molasses + yeast extract. S: (Salt solution; $CaCl_2$ + $MnCl_2$ + $MgCl_2$).

When the obtained microscope images, growth curves and SDS-PAGE results were evaluated together: **(1)** in all media conditions we have seen a gradual increase in toxin production starting from the 24 hours of incubation; **(2)** in all media conditions the highest toxin production was seen in 48th hour samples; **(3)** in all media conditions toxin production in 48 and 72 hours were comparable to each other, although toxin production was slightly higher in 48th hour samples; **(4)** addition of salt solution into the MYM did not have any effect on toxin production; **(5)** toxin production even in no salt added NYSM

was higher compared to MYM and MYSM; **(6)** sporulation in all media conditions were almost similar to each other, yet toxin production varied as mentioned; **(7)** toxin production in NYSM was highest similar to our previous observations.

4.3.3. Effect of whey and salt solution on sporulation and toxin production

Whey is a valuable byproduct of dairy industry. With 6-10 g/L of protein and 46-52 g/L lactose contents it is also a good source of nitrogen and carbon for microorganisms. To minimize cost, it is preferable over yeast extract and peptone which are obtained from overseas producers; and therefore, we wanted to use whey as a carbon and nitrogen source to grow Btk-HD73 and compare toxin production in this new medium to that of NYSM.

To this end Btk-HD73 was first grown in varios whey containing medium derivatives at 30°C with 250 rpm constant shaking for 72 hours (Figure 51).

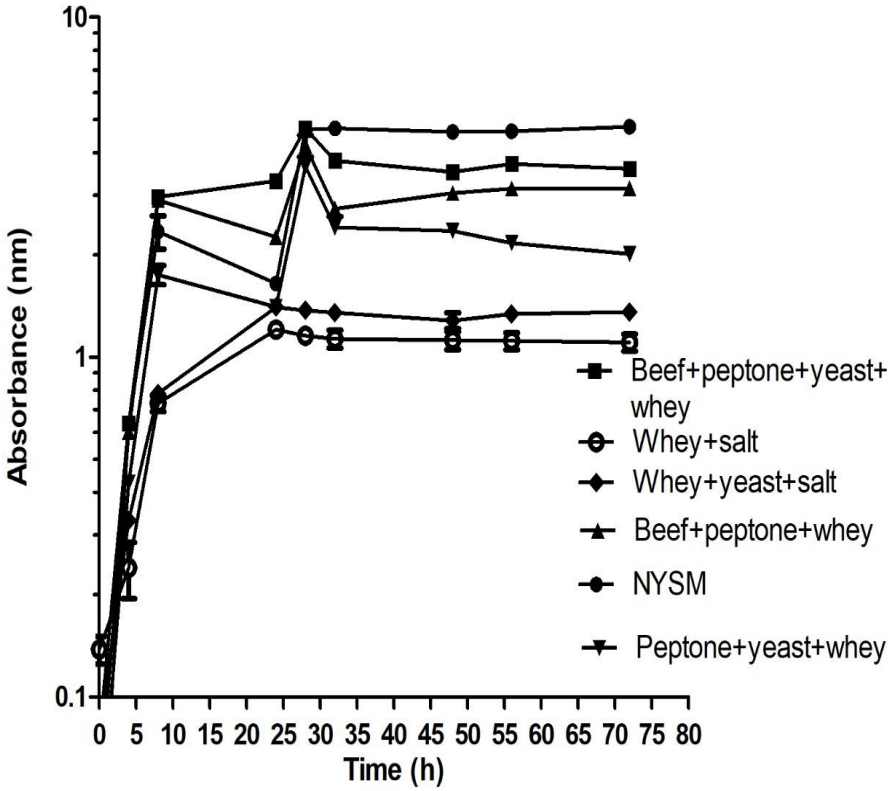


Figure 51 Growth curves of Btk-HD73 in BPYWM, WSM, WYSM, BPWM, PYWM, and NYSM. Cultures were incubated at 30 ° C with 250 rpm constant shaking and the absorbance measurements were taken at 600 nm. BPYWM (beef extract+ peptone+ yeast extract+ whey); WSM (whey+ salt content); WYSM (whey+ yeast extract+ salt content); BPWM: (beef+ peptone+ whey); PYWM (peptone+ yeast extract+ whey); NYSM; (beef extract + peptone + yeast extract + salt content). Salt (Salt solution; $CaCl_2$ + $MnCl_2$ + $MgCl_2$).

When the growth curve was examined, it was noted that: **(1)** the lowest growth was obtained in the whey alone and its salt added medium; **(2)** in all other media addition of whey had a slightly positive effect on growth; **(3)** the highest growth was obtained in NYSM.

During the growth in these media, samples were also collected at every 24 hours to check toxin production (Figure 52 and 53) and sporulation (Figure 54, 55 and 56).

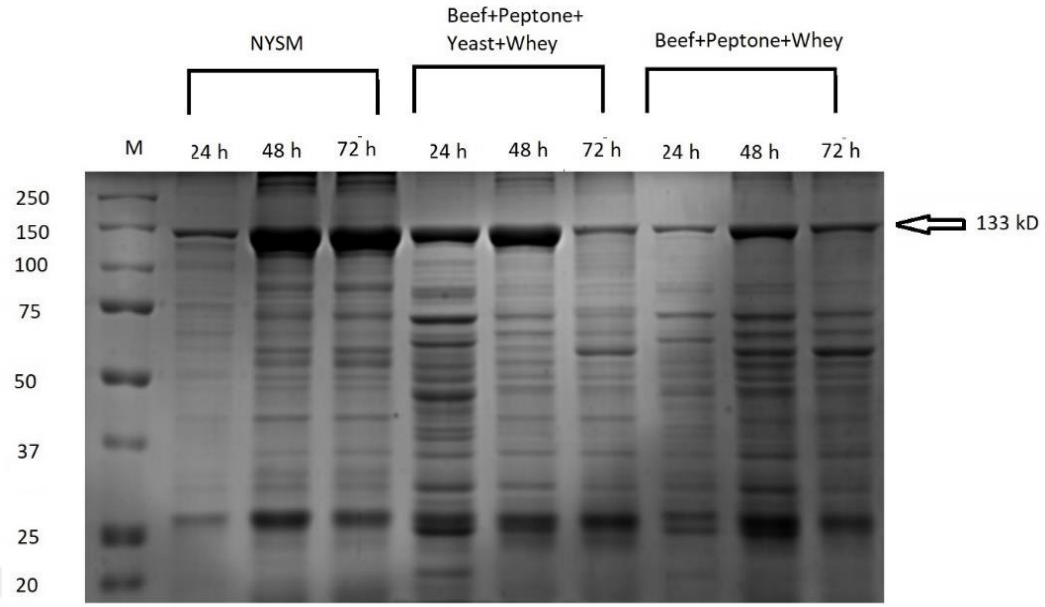


Figure 52 Btk-HD73 total protein SDS-PAGE analysis at 24th,48h and 72th hours in NYSM, BPYWM and BPWM.

The change in protein profile was observed by SDS-PAGE analysis at 24th,48th and 72th hours depending on the growth of HD-73 strain in different media. BPYWM (beef extract+ peptone+ yeast extract+ whey); BPWM: (beef+ peptone+ whey); NYSM; (beef extract + peptone + yeast extract + salt content). Marker (M) next to the left column; values are given in kDa.

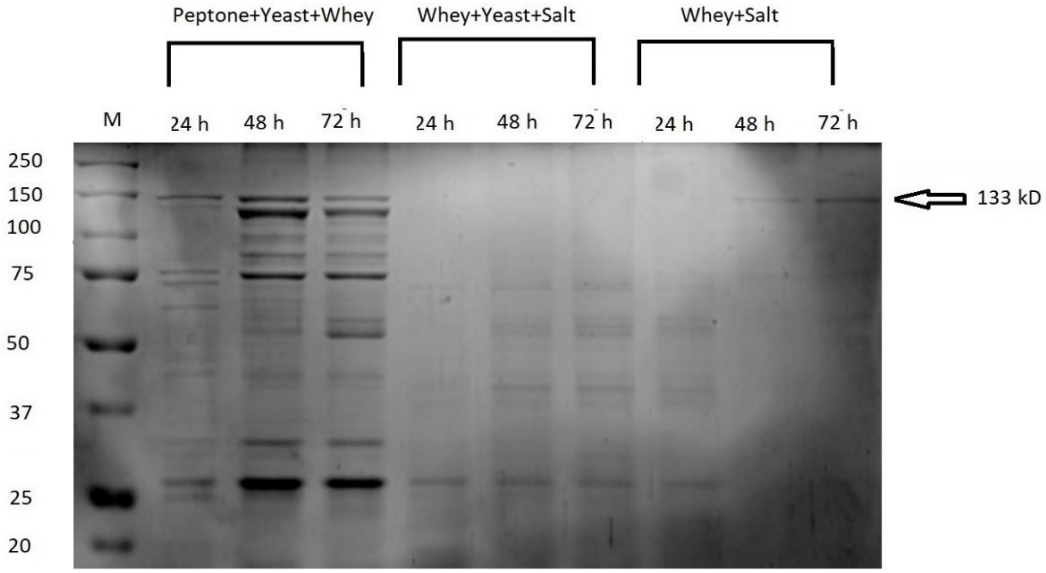


Figure 53 Btk-HD73 total protein SDS-PAGE analysis at 24th,48th and 72th hours in PYWM, WYSM and WSM.

The change in protein profile was observed by SDS-PAGE analysis at 24th,48th and 72th hours depending on the growth of HD-73 strain in different media. WSM (whey+ salt content); WYSM (whey+ yeast extract+ salt content); PYWM (peptone+ yeast extract+ whey. (Salt solution; $CaCl_2$ + $MnCl_2$ + $MgCl_2$). Marker (M) next to the left column; values are given in kDa.

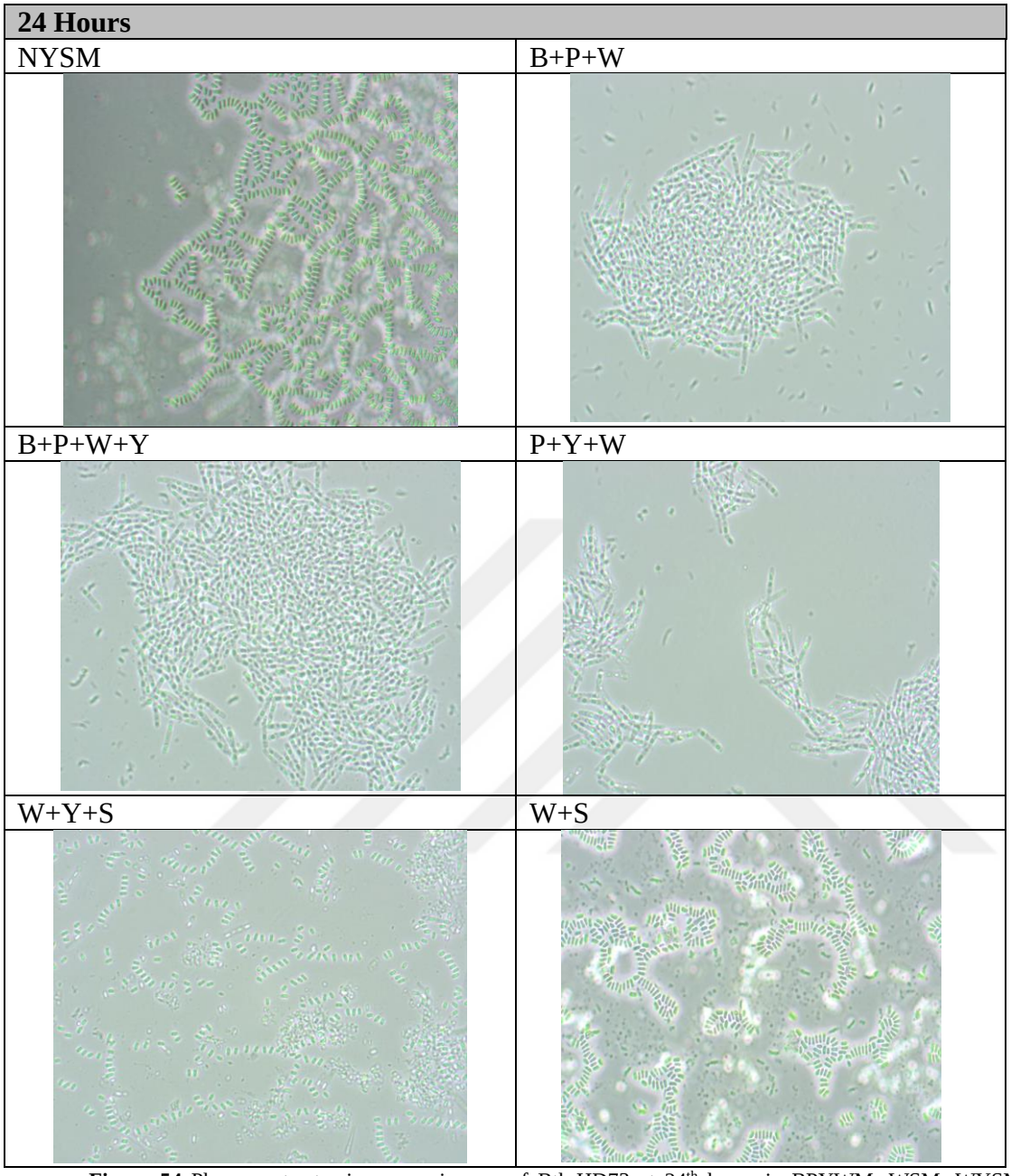


Figure 54 Phase contrast microscope images of Btk-HD73 at 24th hours in BPYWM, WSM, WYSM, BPWM, PYWM, and NYSM.

Culture was incubated in media at 30℃ with 250 rpm shaking for 72 hours. Samples were examined at 100X magnification under a phase contrast microscope at 24th hour. BPYWM (beef extract+ peptone+ yeast extract+ whey); WSM (whey+ salt content); WYSM (whey+ yeast extract+ salt content); BPWM:(beef+ peptone+ whey); PYWM (peptone+ yeast extract+ whey); NYSM; (beef extract + peptone + yeast extract + salt content). S: (Salt solution; $CaCl_2$ + $MnCl_2$ + $MgCl_2$).

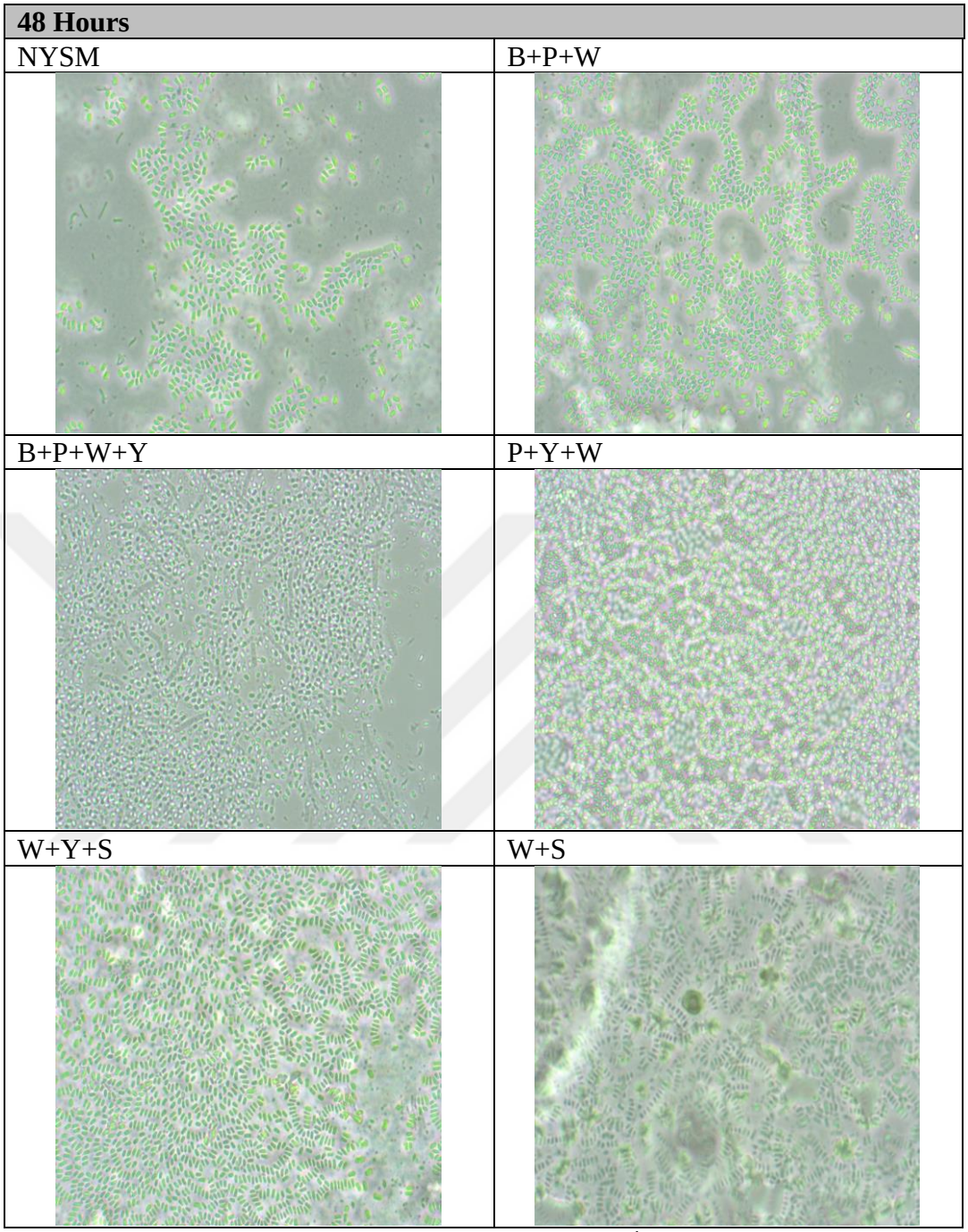


Figure 55 Phase contrast microscope images of Btk-HD73 at 48th hours in BPYWM, WSM, WYSM, BPWM, PYWM, and NYSM.

Culture was incubated in media at 30℃ with 250 rpm shaking for 72 hours. Samples were examined at 100X magnification under a phase contrast microscope at 48th hour. BPYWM (beef extract+ peptone+ yeast extract+ whey); WSM (whey+ salt content); WYSM (whey+ yeast extract+ salt content); BPWM:(beef+ peptone+ whey); PYWM (peptone+ yeast extract+ whey); NYSM;(beef extract + peptone + yeast extract + salt content). S: (Salt solution; $CaCl_2$ + $MnCl_2$ + $MgCl_2$).

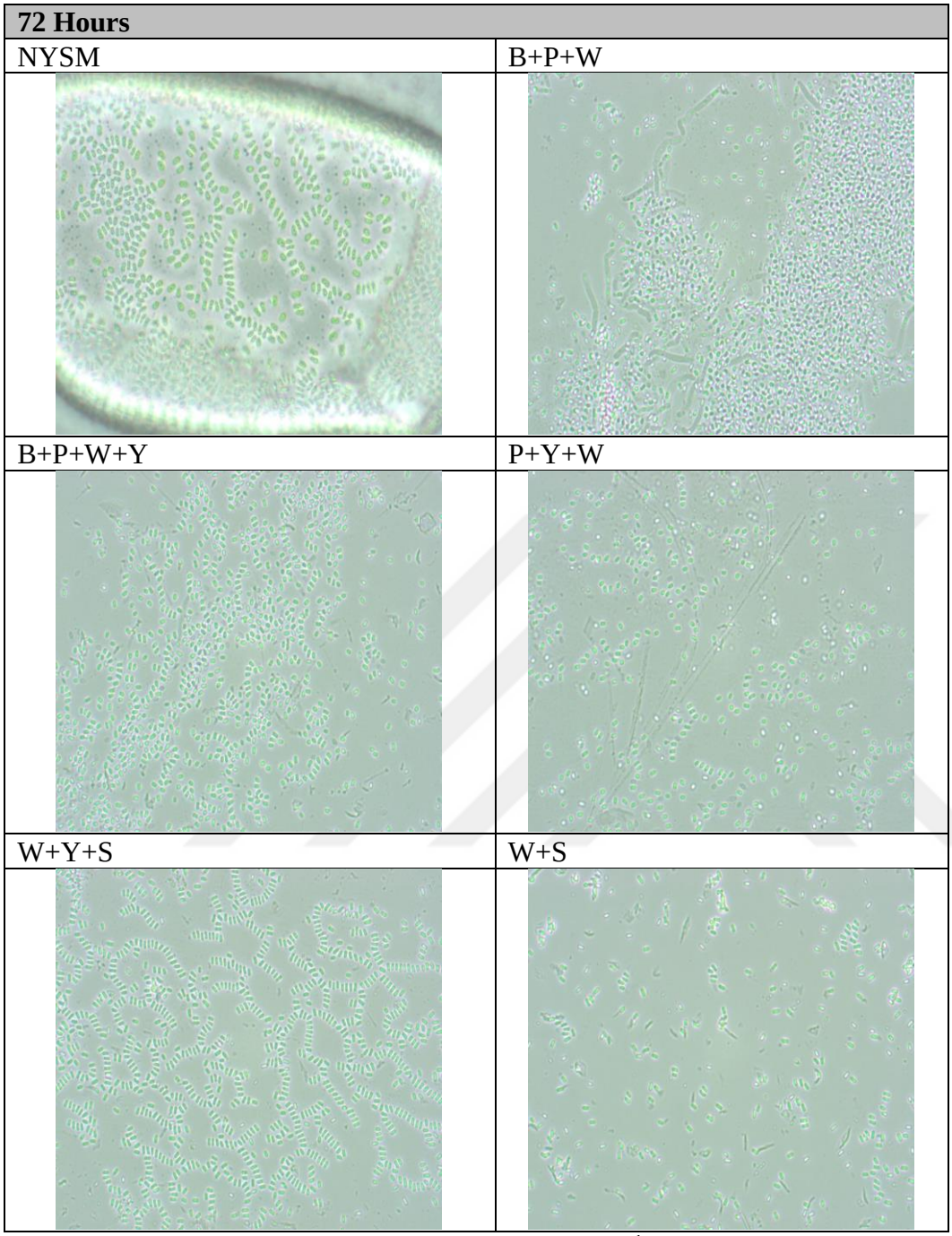


Figure 56 Phase contrast microscope images of Btk-HD73 at 72th hours in BPYWM, WSM, WYSM, BPWM, PYWM, and NYSM.

Culture was incubated in media at 30℃ with 250 rpm shaking for 72 hours. Samples were examined at 100X magnification under a phase contrast microscope at 72nd hour. BPYWM (beef extract+ peptone+ yeast extract+ whey); WSM (whey+ salt content); WYSM (whey+ yeast extract+ salt content); BPWM:(beef+ peptone+ whey); PYWM (peptone+ yeast extract+ whey); NYSM;(beef extract + peptone + yeast extract + salt content). S: (Salt solution; $CaCl_2$ + $MnCl_2$ + $MgCl_2$).

When the results of SDS-PAGE analyses and microscopic observations were evaluated together: **(1)** although we have seen similar sporulation patterns in all media, no visible toxin production was observed in whey alone or its salt added media; **(2)** addition of peptone, yeast extract or beef extract made a positive contribution to toxin production, however, in all these media toxin production was lower compared to that of NYSM.

4.3.4. Effect of medium volume on sporulation and toxin production

Previous experiments were carried out in 50 ml of selected media in a 250 ml flask as given in materials and methods section. Then we wanted to see whether an increase in growth medium volume would have any effect on growth, sporulation and toxin production of Btk-HD73.

To this end we used NYSM as Btk-HD73 had shown highest toxin production in this medium. Btk-HD73 was grown in 250 ml of NYSM in a 500 ml flask at 30°C with 250 rpm constant shaking for 72 hours. No salt added NYM was used as a control. Samples were taken at 48th hour time point to check toxin production using SDS-PAGE (Figure 57) and also sporulation was quantified as CFU/ml in these collected samples.

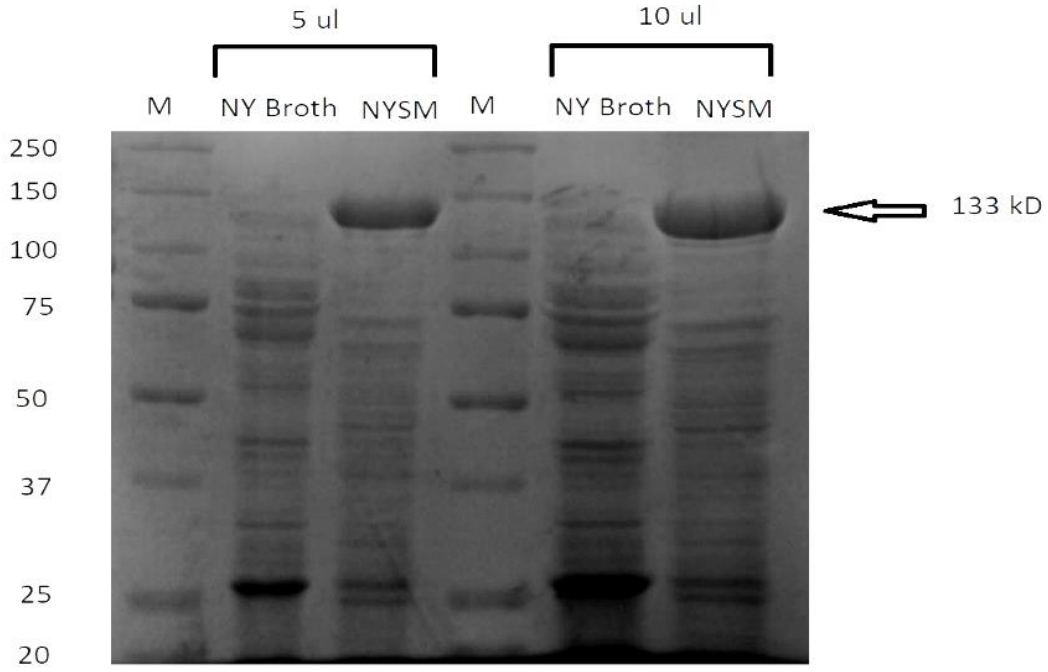


Figure 57 Btk-HD73 total protein SDS-PAGE analysis at 48th hours in 250 ml of NY Broth and NYSM .
The change in protein profile was observed by SDS-PAGE analysis at 48 hours depending on the growth of HD-73 strain in NY Broth and NYSM. 5 µl and 10 µl of samples were loaded into gel for the examination of volume effect.

In 48th hour samples, spore numbers were found to be 4,5±10 x 10^7 CFU/ml in NYM and 18,1±5,0 x 10^8 CFU/ml in NYSM. This indicated an approximate 4-fold increase in spore numbers parallel to our previous microscopic observations.

Toxin production in these conditions were similar to that of lower volumes. In 48th hour samples while there was no visible toxin protein band in NYM, toxin production could be observed in NYSM.

4.4. Correlation of spore numbers with toxin production

During the optimization studies in conventional culture settings we have tried various carbon and nitrogen sources to see their effects on sporulation and toxin production. In these screening experiments we have mainly use SDS-PAGE analysis to check toxin production and microscopic observations to follow sporulation in various samples.

Then we have selected representative media in which we had obtained higher sporulation or toxin production during previous screening experiments (Table 4). Btk-HD73 was grown in 250 ml of this selected media in 500 ml flasks at 30°C with 250 rpm constant shaking for 72 hours. Samples were taken at every 24 hours to check toxin production using SDS-PAGE (Figure 58 and 59) and to follow sporulation both microscopically and doing spore count on agar plates to obtain CFU/ml. In these samples we have also measure δ-endotoxin concentration via Bradford assay (Table 5).

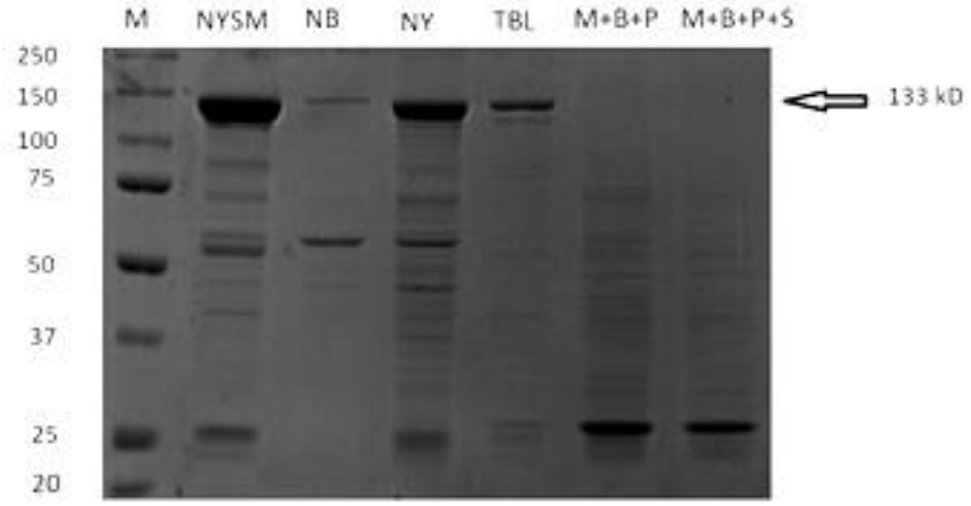


Figure 58 Btk-HD73 total protein SDS-PAGE analysis at 72th hours in NYSM, NB, NY, TBL, MBPM and MBPPSM.

The change in protein profile was observed by SDS-PAGE analysis at 72 hours depending on the growth of HD-73 strain in NYSM, NB, NY, TBL, MBPM and MBPPSM. Marker (M) next to the left column; values are given in kDa. M; Molasses B; Beef extract P, Peptone Y; Yeast (yeast extract) S; Salt (Salt solution; CaCl2 + MnCl2 + MgCl2). To all samples 1,5 x 10^9 CFU/ml spore number used.

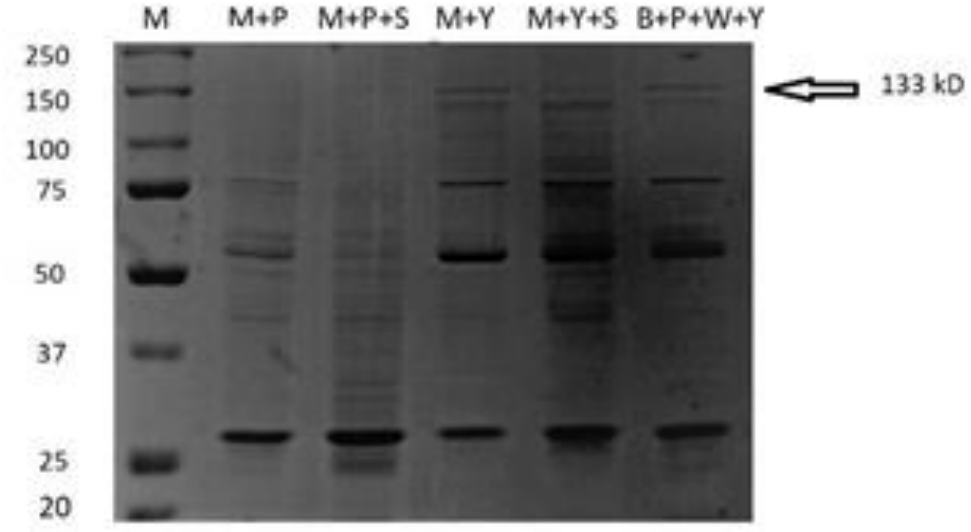


Figure 59 Btk-HD73 total protein SDS-PAGE analysis at 72th hours in MPM, MPSM, MYM, MYSM and BPWYM.

The change in protein profile was observed by SDS-PAGE analysis at 72 hours depending on the growth of HD-73 strain in in MPM, MPSM, MYM, MYSM and BPWYM. Marker (M) next to the left column; values are given in kDa. M; Molasses B; Beef extract P; Peptone Y; Yeast (yeast extract) S; Salt (Salt solution; CaCl2 + MnCl2 + MgCl2). To all samples 1,5 x 10^9 CFU/ml spore number used.

Table 5 A representative standard curve for estimation of protein concentration by Bradford method.

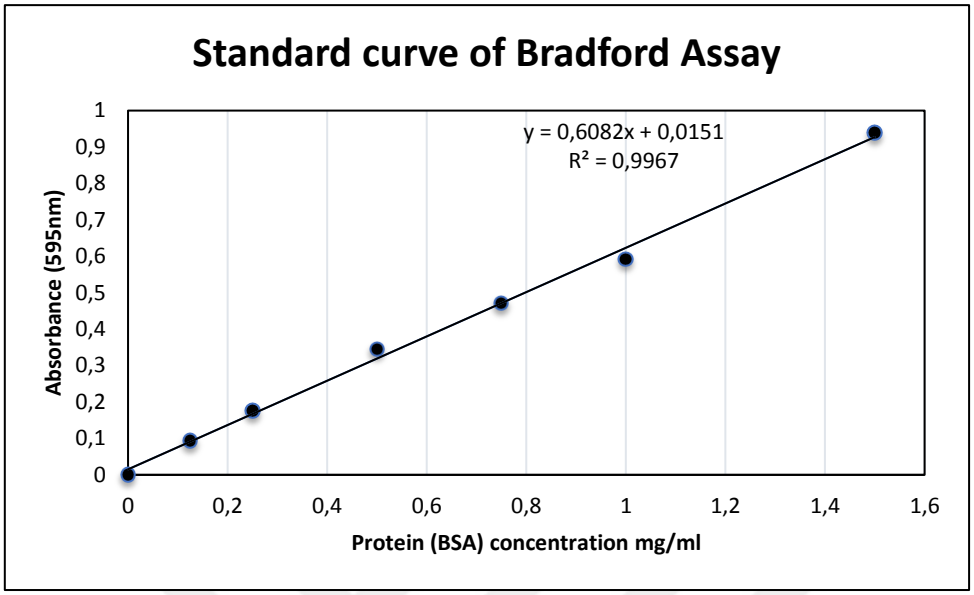
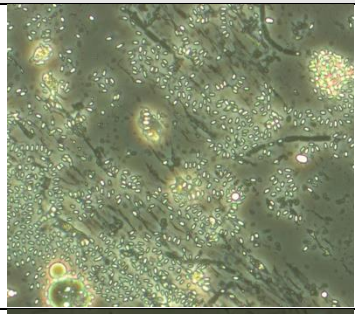
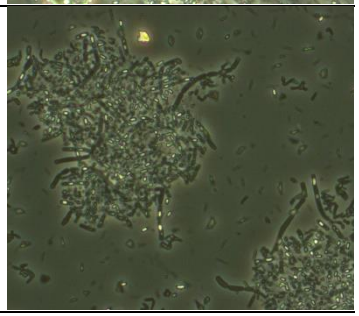
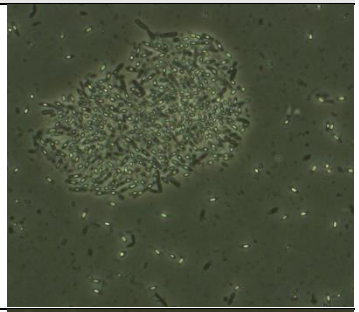
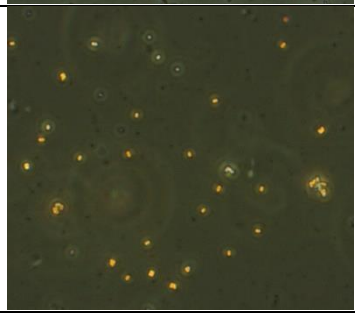
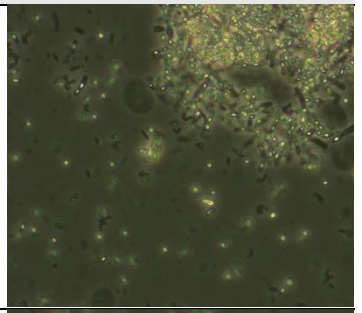
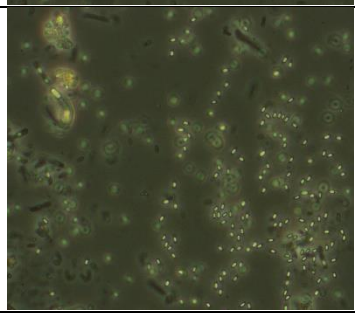
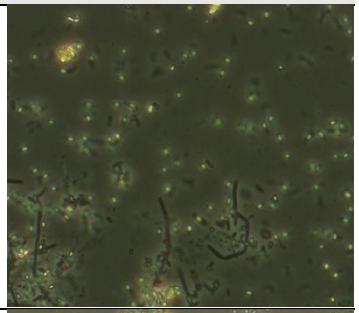
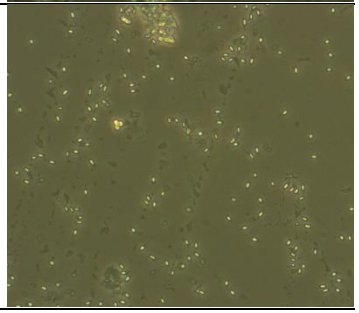


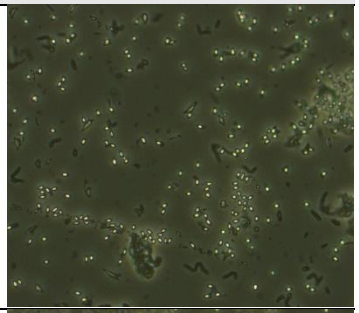
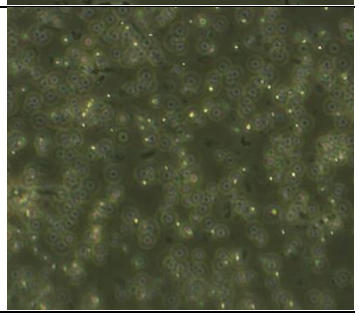
Table 6 Comparison of 11 different media contents with number of spores, δ-endotoxin concentrations, SDS-Page analysis and microscope images.

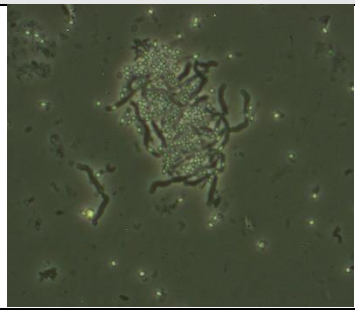
Media	**Spore Count** (CFU/ml) (10^7/ml)	**δ-endotoxin concentration** (mg/ml)	**SDS-Page Analysis**	**Microscope Images**
NYSM	251±1,00	0,6772 ±0,008		
Nutrienth Broth	202±2,00	0,6271±0,001		

Media	**Spore Count** (CFU/ml) (10^7/ml)	**δ-endotoxin concentration** (mg/ml)	**SDS-Page Analysis**	**Microscope Images**
NY	180±0,00	0,6542 ± 0,009		
TBL	188±12,5	0,6583 ±0,002		

Media	**Spore Count** (CFU/ml) (10^7/ml)	**δ-endotoxin concentration** (mg/ml)	**SDS-Page Analysis**	**Microscope Images**
Molasses beef peptone medium	230±15,0	0,6772 ± 0,000		
Molasses beef peptone salt medium	305±15,0	0,6854 ± 0,003		

Media	**Spore Count** (CFU/ml) (10^7/ml)	**δ-endotoxin concentration** (mg/ml)	**SDS-Page Analysis**	**Microscope Images**
Molasses peptone medium	303±2,50	0,6254 ±0,010		
Molasses peptone salt medium	241±1,00	0,6517 ±0,006		

Media	**Spore Count** (CFU/ml) (10^7/ml)	**δ-endotoxin concentration** (mg/ml)	**SDS-Page Analysis**	**Microscope Images**
Molasses yeast medium	345±25,0	0,6386 ±0,003		
Molasses yeast salt medium	265±25,0	0,6451 ±0,009		

Media	**Spore Count** (CFU/ml) (10^7/ml)	**δ-endotoxin concentration** (mg/ml)	**SDS-Page Analysis**	**Microscope Images**
Beef peptone yeast whey medium	270±20,0	0,6501 ±0,023		

When these results examined together: **(1)** it was found that there was more sporulation in the medium containing molasses and yeast extracts; however, toxin production was either not observed or very low in these media. As noted in previous results, sporulation and toxin production did not always show a positive correlation; **(2)** no significant differences were observed in δ-endotoxin concentrations in various media that we had used; **(3)** Btk-HD73 toxin production was highest in NYSM, similar to our previous observations. In these last experiments this was confirmed as we performed spore counts and normalized all SDS-PAGE samples to same spore numbers.

4.5. Culture parameters in bioreactor conditions

Carbon sources, media and salt contents used in optimization studies are presented in Table 1. Our optimization studies in conventional culture conditions showed that Btk-HD73's toxin production was highest in NYSM. For this reason, we continued with this medium for our next step where we optimized growth, sporulation and toxin production parameters in bioreactor settings.

In bioreactor studies the total medium volume was 3 liters and total bioreactor volume was 4.5 liters. During growth at 30°C with 250 rpm agitation, samples were taken every 24 hours for 72 hours (Figure 60). In these samples, toxin production was checked using SDS-PAGE (61) and sporulation was checked using both microscopic observations (Figure 62) and spore counting (Table 7).

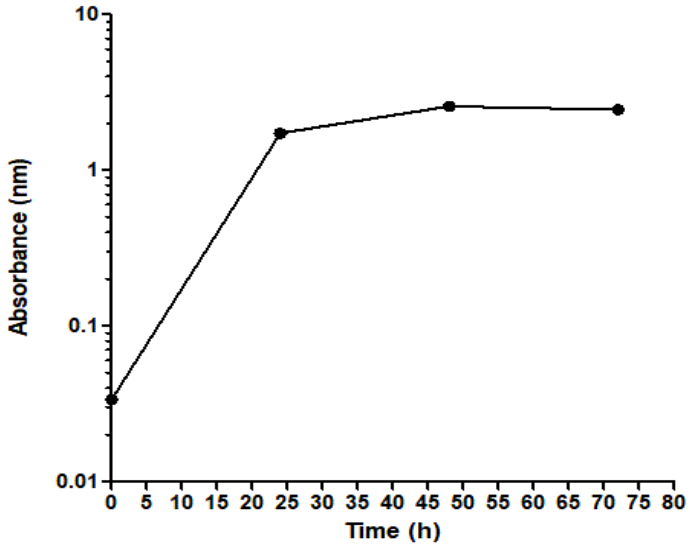


Figure 60 Btk-HD73's growth curve in bioreactor settings.
Btk-HD73 was incubated in a NYSM at 30°C and 250 rpm in bioreactor and absorbance measurements were taken at 600 nm. The pH value was set to 7.

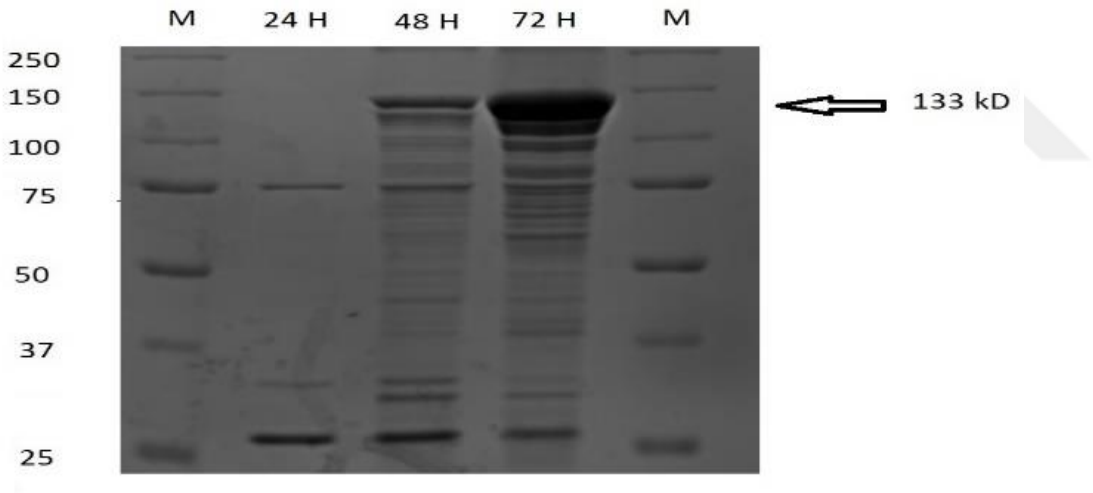


Figure 61 Total protein analysis of Btk-HD73, grown in bioreactor for 72 hours.
The change in protein profile at 24th, 48th and 72th hours was observed by SDS-PAGE analysis. Marker (M) next to the left column; values are given in kDa.

In our conventional culture studies, the highest toxin production was observed after 48 hours of incubation in NYSM. However, the maximum toxin production was observed to be after 72 hours of incubation under bioreactor conditions (Figure 61).

Spores in these samples were also counted (Table 7). According to these results, it was observed that there was a 7-fold increase in the second 24 hours and an approximate 3-fold increase in the third 24 hours.

Table 7 Number of spores obtained from samples taken at different time points in bioreactor environment with NYSM.

Sampling Time	Spore Count
24 Hours	$1{,}0 \times 10^7$
48 Hours	$7{,}5 \times 10^7$
72 Hours	$2{,}1 \times 10^8$

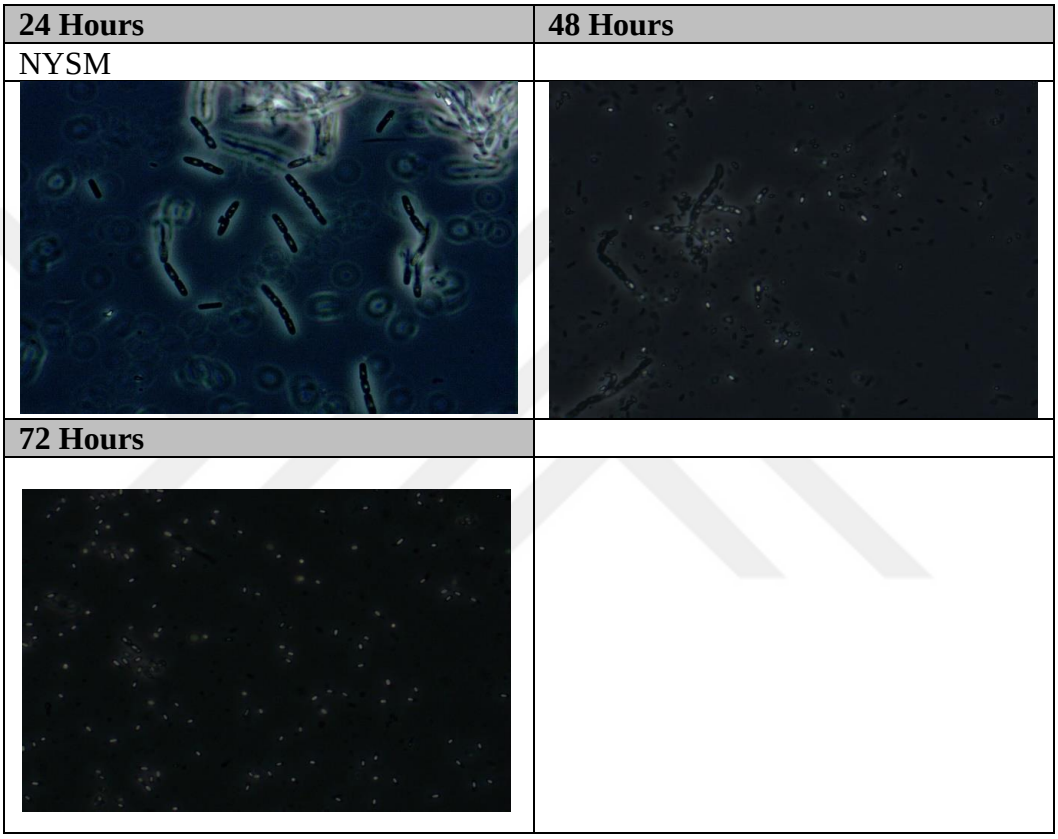


Figure 62 Phase contrast microscope images of Btk HD-73 grown in a bioreactor NYSM for 72 hours. Culture was incubated at 30° C, 250 rpm and fixed pH 7 for 72 hours. For microscopic images, samples taken at 24, 48 and 72 hours were examined with a 100X magnification under a microscope.

Considering spore counts and toxin production results together, it was observed that we had successfully translated conventional culture conditions to bioreactor settings at least for NYSM.

Our studies in the bioreactor environment were continued with different media conditions. First, we compared NYSM with MYM (molasses+ yeast extract) under same conditions for toxin production and sporulation.

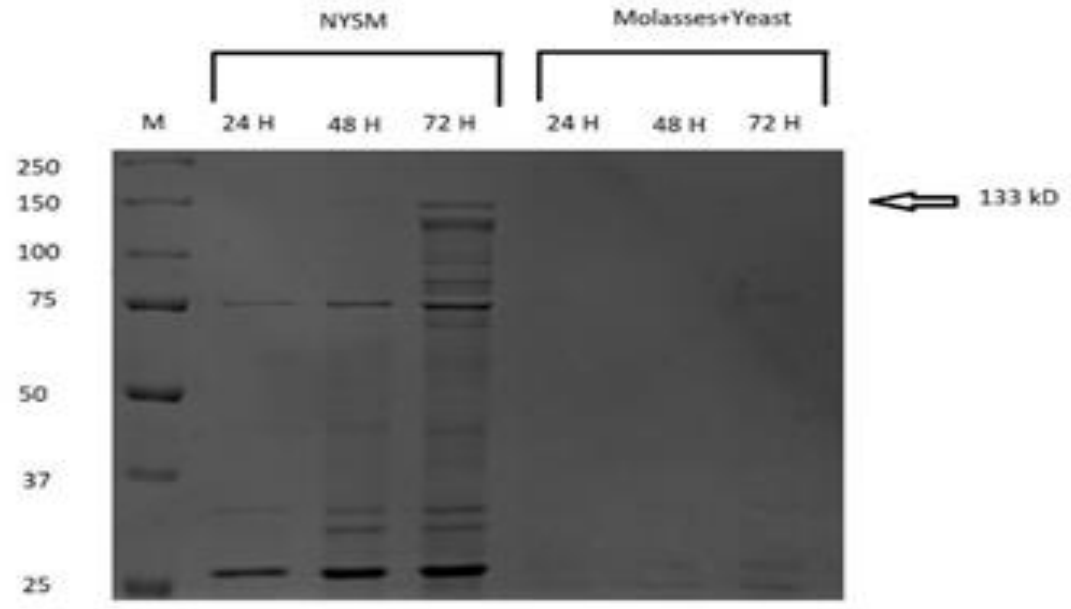


Figure 63 Btk-HD73 total protein SDS-PAGE analysis at 24th, 48th and 72th hours in a bioreactor with 3-liter NYSM and MYM.

Btk-HD73's crystal toxin protein production was visualized at 24th, 48th and 72 hours th time points using SDS-PAGE analysis during growth in a bioreactor NYSM and MYM at 30° C, 250 rpm and fixed pH 7 for 72 hours. Marker (M) next to the left column; values are given in kDa.

Table 8 Number of spores obtained from samples taken at different times in bioreactor environment with MY medium.

Sampling Time	**Spore Count**
24 Hours	$(3,0 \pm 0,5) \times 10^7$
48 Hours	$(4,0 \pm 1,0) \times 10^7$
72 Hours	$(3,0 \pm 1,0) \times 10^7$

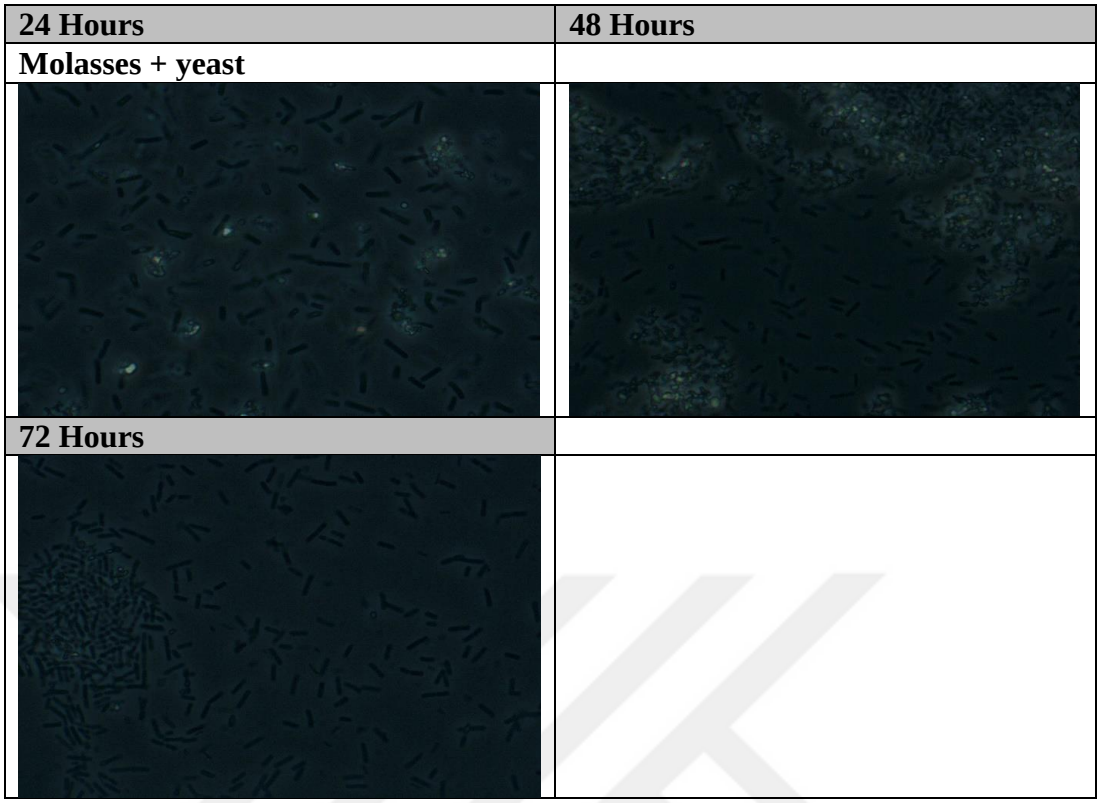


Figure 64 Phase contrast microscope images of Btk-HD73 in bioreactor.
Culture was incubated at 30° C, 250 rpm and fixed pH 7 for 72 hours. For microscopic images, samples taken at 24th, 48th and 72th hours were examined with a 100X magnification under a microscope.

It was observed that, similar to conventional culture studies, Btk-HD73 did not produce toxin in the presence of molasses, although spore numbers were comparable to that of produced NYSM.

MYM did not contain any salt unlike NYSM. We then wanted to see whether salt addition would have any positive effect on toxin production. In our previous results, addition of salt to MYM in conventional culture conditions hadn't had any positive effect on toxin production. To this end Btk-HD73 was grown under the same conditions in MYSM (molasses+ yeast extract+ salt) for 72 hours in bioreactor and samples were collected every 24 hours to check toxin production and sporulation.

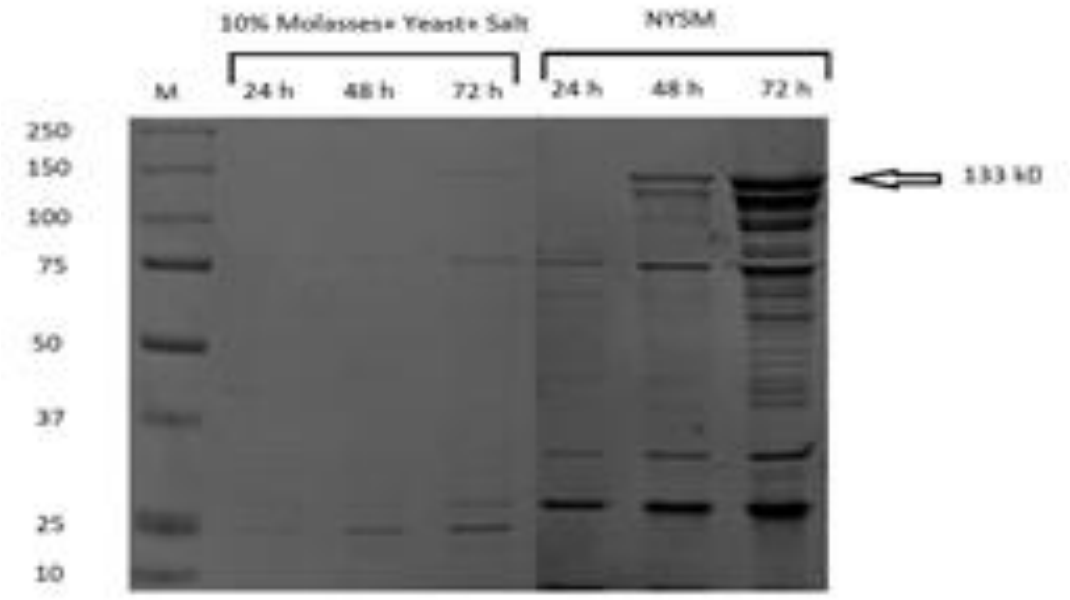


Figure 65 Btk-HD73 total protein SDS-PAGE analysis at 24th, 48th and 72 hours in a bioreactor with 3-liter MYS medium.

Btk-HD73's crystal toxin protein production was visualized at 24th, 48th and 72th hours' time points using SDS-PAGE analysis during growth in bioreactor NYSM and MYSM at 30° C, 250 rpm and fixed pH 7 for 72 hours. Marker (M) next to the left column; values are given in kDa.

Table 9 Number of spores obtained from samples taken at different times in bioreactor environment with MYS medium.

Sampling Time	**Spore Count**
24 Hours	$2{,}0 \times 10^7$
48 Hours	$2{,}5 \times 10^7$
72 Hours	$5{,}0 \times 10^7$

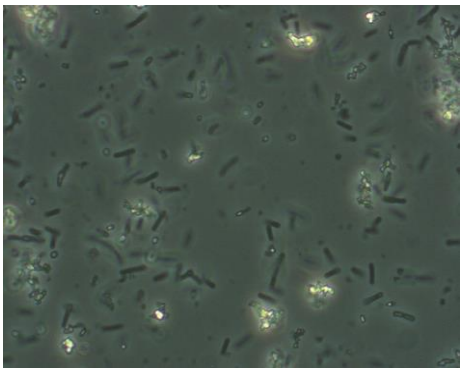
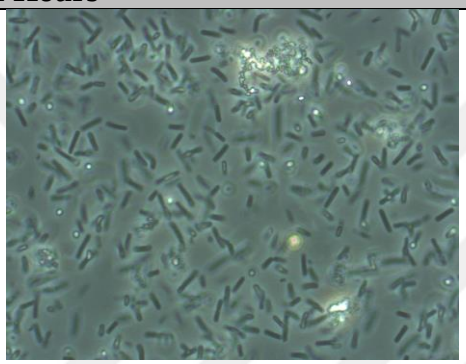
24 Hours	48 Hours
Molasses + yeast + salt	
	
72 Hours	
	

Figure 66 Phase contrast microscope images of Btk-HD-73 in bioreactor with MYS medium. Culture was incubated at 30° C, 250 rpm and fixed pH 7 for 72 hours. For microscopic images, samples taken at 24th, 48th and 72th hours were examined with a 100X magnification under a microscope.

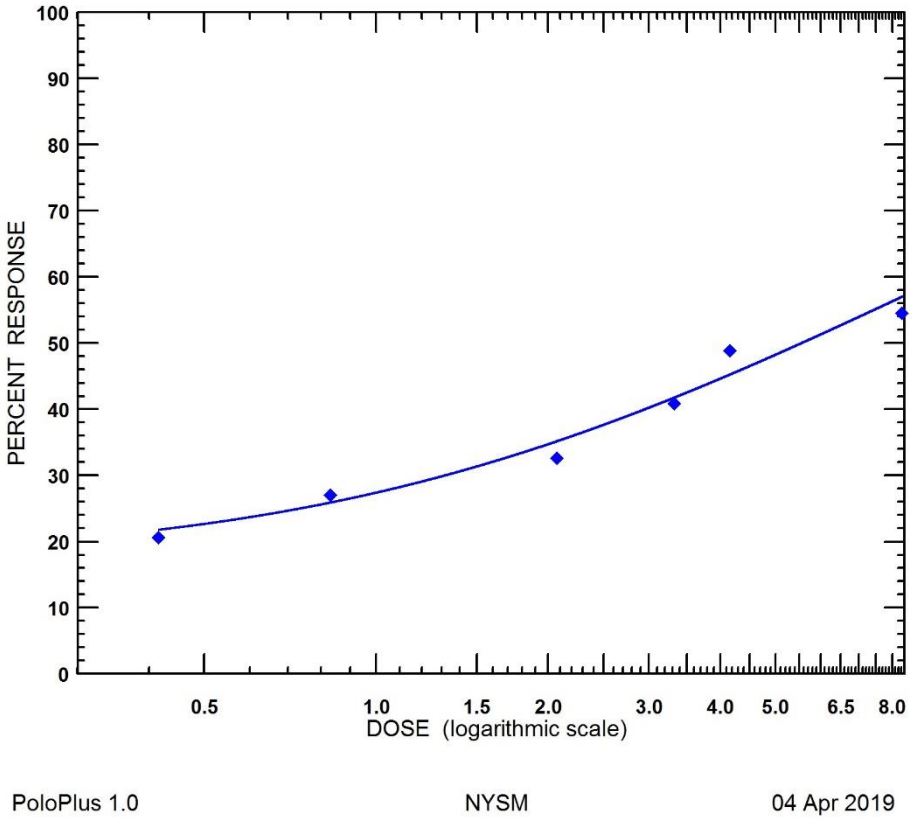
We have observed that salt addition did not cause any significant increase in toxin production. Spore numbers were also similar to that of MYM.

As a result of studies in conventional culture and bioreactor environment, NYSM showed the most successful result and it was used in biological activity experiments on *T. absoluta*.

4.6. Bioassay Results

The LC_{50} value, 95% confidence limits, standard errors, the slopes of the regression lines and $\chi 2$ signifcance tests, were estimated by probit analysis using PoloPlus 1.0 sofware (LeOra Sofware, California, United States).

Figure 67 Log-dose probit mortality data for *T. absoluta* populations tested with Btk-HD73 grown in NYSM. Analysis was performed in each laboratory.



Mortality data is presented in Figure 67. Mortality data from concentration-response assays fitted the Probit model ($\chi 2$ not significant, $P > 0.05$). The LC_{50} is 0.925 µl/ L^{-1} which means required dose to kill half of the population.

The slope value (1.232 ± 0.430) stated a homogeneous population. The slope of the logarithmic dose-probit line indicated the change in the reaction to the toxic substance (Hoskins, 1960). If the slope is perpendicular, a slight change causes a large change in the death rate. However, if this line is flat, it can be said that the difference in insects is less and higher doses should be used to achieve more deaths.

Table 10 Toxicity of Btk-HD73 grown in NYSM to 2nd-instar larvae of *T. absoluta.*

Insecticide	n[a]	Slope ± SE[b]	LC_{50} (CI 95%)[c]	χ2(DF)[d]
NYSM	253	1.232 ± 0.430	0.925	0.533

[a] Total number of insects bioassayed.
[b] Standard Error.
[c] Microliter of active ingredient per liter water.
[d] Chi-square and Degree of Freedom.

Table 11 Relative toxicity of Btk-HD73 grown in NYSM and Dipel to 2nd-instar larvae of *T. absoluta.*

	NYSM	**Dipel (Commercial)**
Spore Count	32 x 10^5 CFU / 100 ml	32 x 10^5 CFU / 100 ml
% Mortality (Fresh Preparation)	%100	%100
% Mortality (4 Months old preparation)	%50	%90

In the studies related to the development of commercial formulation, optimizations were made with NYSM in bioreactor settings and then biological activity tests were performed on *T. absoluta* larvae. In these experiments, significant results were obtained in terms of toxin's efficacy and stability. The first bioassay trial with fresh samples were showed near 100% death on larvae both for Dipel and NYSM formulation. However, in the last experiment carried out about 4 months after the production of the sample, 50% death rate was observed for NYSM formulation; and this was 90% for Dipel.

5. DISCUSSION

5.1 Effect of molasses on sporulation and toxin production

Bacillus thuringiensis is a Gram-positive bacterium that produces entomopathojenic crystal proteins simultaneously with sporulation. *B. thuringiensis* have been used as a biocontrol agent for many years. Sporulation and toxin production are directly related to growth parameters like carbon/nitrogen source, temperature, aeration and pH. Addition to this, a formulation of the toxin containing spores of this bacterium to be used in the field, should contain some UV and cryoprotectants for a successful field application. For this reason, growth optimization and formulation studies are crucial to obtain a succesfull product. Optimization studies depend on improvement of the toxin synthesis and sporulation with the use of different carbon, nitrogen and salt source in both conventional and bioreactor media. In literature there are many examples for the use of alternative raw materials: molasses (Amin 2016), starch, soya bean, yeast (Dhouha Ghribi et al., 2007).

In this study, we aimed to optimize medium conditions of *B. thuringiensis* subsp. *kurstaki* HD-73 strain for maximum Cry1Ac toxin production both in conventional culture and bioreactor seetings. To this end we used various carbon, nitrogen and salt sources. Among various carbon sources tried, molasses was especially important, being a byproduct of sugar industry, it is easy to find and cheaper compared to yeast extract, beef extract and peptone. Growth medium cost might become an important cost factor during the production of the targeted formulation at the end.

In our studies where we used molasses as the sole or an additional carbon source in the growth medium, we observed successful sporulation in all cases; however, we have failed to obtain Cry toxin production. Btk-HD73 did not produce toxin in these growth media, but only spores. In *B. thuringiensis*, crystal protein synthesis are controlled by different mechanisms; Sporulation-dependent *cry* gene expression and Non-sporulation dependent *cry* gene expression (Schnepf, Crickmore, Rie, et al., 1998). Although Cry1A toxin family gene expressions are known to be as sporulation dependent, clearly there is another pathway according to our experimental data. Additionally, there isn't enough literature on whether environmental signals could regulate toxin expression or not.

One possible explanation for not seeing toxin production in molasses containing growth media, is carbon catabolite repression in *Bacillus* species (Dhouha Ghribi et al., 2007; Görke & Stülke, 2008; Stülke & Hillen, 2002; Stulke et al., 1995; Zouari, Ali, & Jaoua, 2002). This response occurs when concentrations of assimilated carbon sources is high in growth medium and one of the major limitation of endotoxin production (Dhouha Ghribi et al., 2007). The HPr proteins and HPr kinase pathway is the central sensor in catabolite repression especially in *Bacillus* family and some other Gram-positive bacteria (Stulke et al., 1995). However, in *B. thuringiensis*, HPr kinase was not shown to repress regulation of endotoxin synthesis. In this respect, the repressive effect of glucose in fermentation medium, can be maintain by controlling dissolved oxygen in medium in a bioreactor (Dhouha Ghribi et al., 2007).

5.2. Effect of salt solution on sporulation and toxin production

As previously mentioned spore and toxin production can be improved with the use of suitable media conditions (D. Ghribi, Zouari, & Jaoua, 2005). Apart from carbon and nitrogen sources; potassium (K_2HPO_4) and various metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}) should also be considered as they create much needed stress conditions for spore formation. Especially Mn^{2+} is the most substantial co-regulating metal. Ca^{2+} also positively affects sporulation and is essential both for growth and delta endotoxin synthesis; whereas, Zn^{2+} has been shown to decrease sporulation (Içgen et al., 2002).

To this end, a salt solution ($MnCl_2$, $CaCl_2$ and $MgCl_2$) that was mainly used in NYSM was added to different prepared media to observe its effect on sporulation and toxin production.

Our results indicated that the addition of salt solution in molasses containing media did not have any effect on toxin production; but just on sporulation in few cases. A salt effect on the spore formation was only observed in molasses beef peptone salt (MBPS) and NYSM media. Addition of salt to MBP medium increased spore production by around 32%. On the other hand, the increase in the number of spore was found to be approximately 38% in NYSM medium in comparison with NY broth. Furthermore, the enhancing effect of salt on toxin synthesis can be clearly seen when the thickness of

obtained protein bands is examined for these media. In fact, when all results were examined, it was observed that the sporulation and toxin production was highest in the NYSM. As mentioned in previous studies, although crystal protein synthesis is known as sporulation-dependent phenomenon, these processes may not be regulated by metals. This situation have not yet been clearly demonstrated (Içgen et al., 2002).

5.3. Effect of whey and salt solution with different carbon sources on sporulation and toxin production

Whey powder is a good source of carbon and nitrogen. Also, being a byproduct of dairy industry, it is easy to obtain and cheaper compared to yeast/beef extract and pepton. For this reason, whey powder was also tested together with molasses in various combinations and concentrations in Btk-HD73 growth media.

Only in one combination, beef peptone yeast whey, we obtained a comparable success to NYSM. But again, salt addition did not increase toxin production. As the toxin production was the formost important parameter and this content did not differ much in terms of cost compared to the NYSM medium, we did not use this medium in the bioreactor trials.

5.4. Correlation of spore numbers with toxin production

In our experiments we first counted spores from plates inoculated directly from cultures without any process just by serial dilution. However, this caused some scattered spore numbers even from the same source (data not shown). Use of 3 mm glass beads, low concentration of non-ionic detergent addition, heating to 80° C, etc. are among few processes options in the literature to overcome this effect.

After several trials we could successfully adopt a procedure. First we added an initial shaking step at 1.000 rpm for 15 minutes with glass beads to separate all possible coagulated spores from each other. Then we used an incubation period at 80°C for 15 minutes to kill any vegetative cells present in the sample. After this last stage, serial dilutions can be prepared to spread on target agar plates.

By using this method, we could overcome spore aggregation and vegetative cell contamination problems in our samples.

5.5. Transfer of conventional culture parameters to bioreactor settings

Our optimization studies in conventional cultures showed that NYSM as the best medium both for sporulation and toxin production for Btk-HD73. Then we used this medium in bioreactor settings preserving growth parameters like temperature and agitation similar. In our growth experiments in bioreactor we could be able to obtain high spore and toxin production with NYSM. The only difference was in the incubation time. In bioreactor settings the time point where we had obtained highest toxin production shifted from 48 hours to 72 hours compared to conventional cultures. Considering the possible effect of the volume change, bacteria would need more time to reach the same density and therefore this was considered to be within normal limits.

In a bioreactor it is possible to keep certain growth parameters constant or at desired concentrations during the growth, which is impossible in conventional cultures. One of the important growth parameters for *B. thuringiensis* is pH. In our conventional culture experiments, the pH could not be fixed and exceeded 9 according to our measurements. SDS-PAGE analyses of these first growth experiments showed proteins bands between 50 and 65 kDa (Figure 67), instead of an expected 133 kDa band for Cry1Ac toxin protein. It is known that the 133 kDa Cry1Ac protoxins are activated in the alkaline environment and give 65 kDa protein bands (Du & Nickerson, 1996). We thought that the alkaline environment conditions caused by the pH increase could be the reason for these results, and the protoxins may had been transformed into active form in the medium.

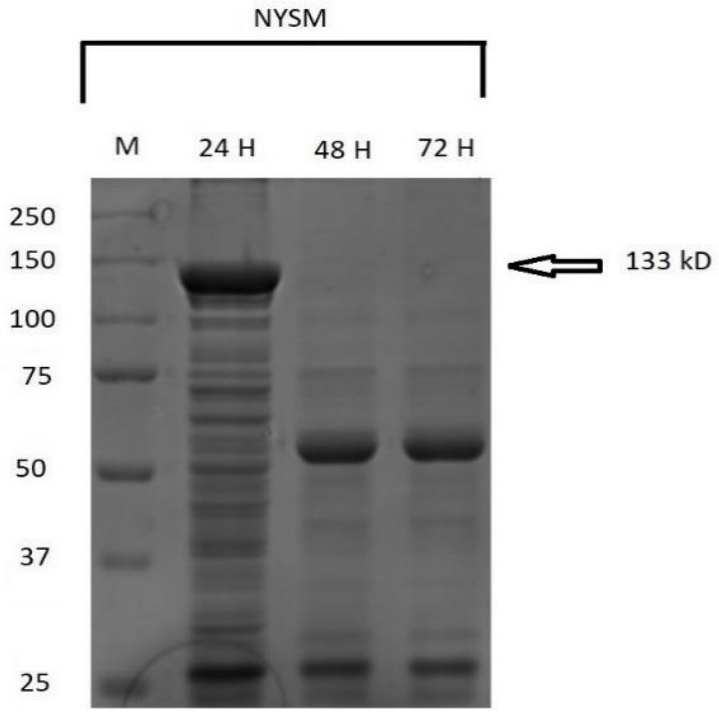


Figure 68 Btk-HD73 total protein SDS-PAGE analysis at 24th, 48th and 72th hours in bioreactor with NYSM. Btk-HD73's crystal toxin protein production was visualized at 24th, 48th and 72th hours' time points using SDS-PAGE analysis during growth in a bioreactor with 3 liter NYSM at 30° C, 250 rpm for 72 hours. Marker (M) next to the left column; values are given in kDa.,

In our regular procedures in the bioreactor, as the pH was kept constant at 7, we did not observe this 60 kDa band, but just the expected 133 kDa band for the protoxin.

5.6. Susceptibility of *T. absoluta* to *B. thuringiensis* Cry toxins

In our first experiments, we obtained similar larval killing results both for our preparation and the DİPEL – the commercial product containing *B. thuringiensis*, with an almost 100% mortality rate. In our later experiments, however, the killing rate for DİPEL continued in the 80-90% range, while the activity of our preparation decreased to 50%. In these later bioassays we used the same preparation that had been prepared almost 4 months ago. This efficacy decrease can be explained by the sensitivity of *B. thuringiensis* Cry toxins to the Ultra Violet (UV) radiation, temperature, presence of oxidative agents and other environmental conditions (Jallouli, Sellami, Sellami, & Tounsi, 2014; Maghsoudi & Jalali, 2017; Sanchis et al., 1999). They are rapidly inactivated through generation of free radicals then oxidation of amino acids and destruction of tryptophan and histidine residues (Sanchis et al., 1999)(Jallouli et al., 2014). This disadvantages of the formulation could be surpassed by adding UV protectants (Maghsoudi & Jalali, 2017)

like nanomaterials and organic compounds, also by the use of encapsulation (Jallouli et al., 2014). Since DİPEL is a commercial product, it is sold in lyophilized form and formulated with these various materials; therefore, no such activity loss was observed.

6. CONCLUSION

In this thesis work we optimized growth and toxin production parameters of *Bacillus thuringiensis* subsp. *kurstaki* HD-73 both in conventional culture and bioreactor settings. In our experiments, we observed highest sporulation and toxin production in NYSM, compared to all other growth media tested, both in conventional culture and bioreactor settings.

The cost for growth media components like yeast and beef extract, is a major limitation factor for a production facility in Turkey; as almost all these components are exported. To this end we have also tried alternative carbon and nitrogen sources like molasses and whey powder. These alternative sources are byproducts of sugar and dairy industries, respectively; and therefore, they are cheaper and easier to obtain. In media containing these alternative sources, although the sporulation was comparable that of NYSM, the toxin production could not be observed.

Biological activity tests were also performed on *Tuta absoluta* larvae using *B. thuringiensis* subsp. *kurstaki* HD-73 spores containing Cry1Ac toxins. In these biological assays LC_{50} value was calculated and a 100% death rate was achieved with our fresh preparations. These preparations, however, showed an activity decrease over time indicating the necessity for further formulation studies to achieve certain level of protection for its ingredients.

In future studies, these preparations containing pure culture will be formulated with various substances and lyophilized to improve its efficiency and stability.

7. REFERENCES

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