# IDENTIFICATION AND CHARACTERIZATION OF A NEW MOSQUITOCIDAL *BACILLUS SPHAERICUS* AND ITS TOXIN PROTEINS

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# ABSTRACT


## IDENTIFICATION AND CHARACTERIZATION OF A NEW MOSQUITOCIDAL *BACILLUS SPHAERICUS* AND ITS TOXIN PROTEINS

The bacterial insecticides have been used for the control of vector mosquitoes for more than five decades. Some strains of *Bacillus sphaericus* produce a highly toxic protein called binary toxins during sporulation. These toxins are composed of toxic BinA (41.9 kDa) and receptor binding BinB (51.4 kDa) proteins and show toxicity against mosquito larvae which vectors of malaria, dengue fever, and yellow fever. In this study, a new mosquitocidal *B. sphaericus* (MBI5) strain is identified. The toxicity of MBI5 is tested in laboratory bioassay that was able to kill the larvae of *Culex pipiens*. Bioassay test results showed that MBI5 was able to kill the larvae, but not pupa and adults of *Culex pipiens*.

The BinA and BinB genes were cloned into pET-16b and expressed in *Escherichia coli* DH5α. The active form of BinA and BinB proteins were purified from the *E. coli* BL21. The purified BinA protein showed $LC_{50}$ dose 206,8 ng $ml^{-1}$ at 8 h against *Culex pipiens* larvae. The BinB protein was reported as non-toxic against *Culex*. The 1:1 molar ratio combination of BinA and BinB proteins showed $LC_{50}$ dose 379,7 ng $ml^{-1}$ at 8 h against *Culex pipiens* larvae.

The results showed that newly isolated and identified *B. sphaericus* MBI5 strain can be used as a potential bacterial insecticide against laves of mosquito species.

# ÖZET


## SİVRİSİNEK ÖLDÜRÜCÜ YENİ BİR SUŞ OLAN *BACILLUS SPHAERICUS* VE TOKSİN PROTEİNLERİNİN TANIMLANMASI VE KARAKTERİZASYONU

Yaklaşık 50 yıldan fazla süredir bacteriyel insektisitler sivrisineklerin kontrolü için kullanılmaktadır. Bazı *B. sphaericus* suşları sporulasyon aşamasında ikili toksinler olarak bilinen yüksek toksin olan proteinleri üretirler. Bu toksinler BinA (41,9 kDa) ve reseptör bağlanma proteini olan BinB (51.4 kDa) proteinlerinden oluşurlar. Bu proteinler sıtma, dang humması, sarı humma gibi hastalıkların taşıyıcısı olabilen sivrisinek larvaları üzerinde toksik etki gösterir. Bu çalışmada kapsamında sivrisinek larvaları üzerinde toksik etkiye sahip yeni bir *Bacillus sphaericus* (MBI5) suşu tanımlanmıştır. Yapılan laboratuvar biyoassay testlerinde test edilen MBI5 suşunun *Culex pipiens* larvalarını üzerinde yüksek düzeyde toksik olduğu, ancak pupa ve erginler üzerinde öldürücü olmadığı saptanmıştır.

BinA ve BinB genleri pET-16b plasmidine klonlandı ve *Escherichia coli* DH5α.suşunda eksprese edildi. BinA ve BinB proteinlerinin aktif formları *E. coli* BL21 suşundan pürifiye edildi. Pürifiye edilmiş BinA proteinin $LC_{50}$ değeri *C. pipiens* larvasına karşı 8. saatte 206,8 ng $ml^{-1}$ olarak bulundu. BinB proteininde *Culex* larvalarına karşı bir toksik etki gözlenmedi. 1:1 oranında hazırlanmış BinA ve BinB protein karışımının *Culex pipiens* larvasına karşı 8. saatte $LC_{50}$ değeri 379,7 ng $ml^{-1}$ olarak kaydedildi.

*B. sphaericus* (MBI5) suşunun, sivrisinek türlerinin larvalarına karşı etkili yeni bir bakteriyel insektisit olduğu ilk defa bu çalışma ile ortaya konulmuştur.

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| | |
|---|---|
| BS | *Bacillus sphaericus* |
| BSA | Bovine serum albumine |
| DENV | Dengue Virus |
| *E. coli* | *Esherichia coli* |
| IPTG | Isopropyl β-D-1-thiogalactopyranoside |
| LB | Luria-Bertani Medium |
| MCS | Molecular Cloning Site |
| PCR | Polimerase Chain Reaction |
| RNA | Ribonucleic acid |
| TAE | Tris Aceteta EDTA |
| WHO | World Health Organization |
| WNV | West Nile Virus |

# 1. INTRODUCTION

Infectious diseases have always accompanied humans, animals, plants, and goods in their travels. The rapid expansion of global trade and transportation since 1700 has been accurately associated with the spread of vector-borne diseases such as yellow fever and malaria. For instance, Dutch Elm disease is originally from Asia and probably transmitted to the United States on a shipment in 1930s and devastated American elms in forest on city streets [1, 2].

Infectious diseases transmitted by insects to humans and other animal vectors, which have been associated with human illness and death for decades. In the 17th through early 20th centuries, humans suffered much pain from vector-borne disease than all other causes combined [3]. Today, the integrated global economy has accelerated the transmission of animal and plant products, which causes the pathogens and their vectors to new hosts and geographic ranges. Presented with these view, several vector-borne diseases can be considered more dangerous than 100 years ago and this is serious threats to public health [4].

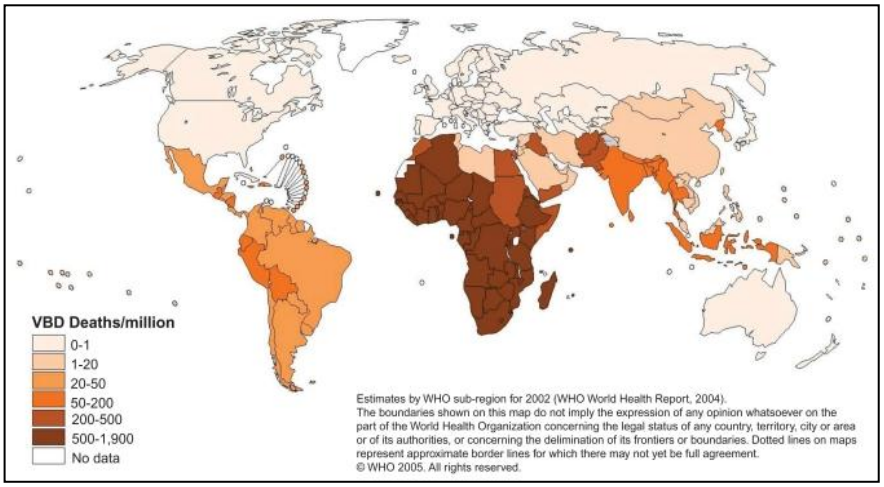


Figure 1.1. Deaths from vector-borne diseases around world [5]

In the early 20th century, some diseases such as malaria, yellow fever, and dengue increased quickly where mosquitoes bred, and eventually to the use of pesticides, which reduced populations of these disease vectors. Today, vector borne diseases are once again a worldwide concern and a significant cause of human morbidity and mortality, especially for Africa and South America (Figure 1.1) [5].

The considerable economic, ecological, and public health impacts of vector-borne diseases are expected to continue. The works about these diseases have been focused on biology, and in particular about the complex biological and ecological relationships that exist among pathogens, vectors, hosts, and their environments [6].

## 1.1. MOSQUITO-BORNE DISEASES

Mosquitoes are important vectors of several viral (Dengue Fever, West Nile Virus (WNV), Yellow Fever), protozoa (Malaria) related diseases. Not only humans are the main target of mosquitoes carry diseases, mosquitoes also afflict and transmit to some animals such dogs, horse etc. Besides, these diseases cause mortality and morbidity among humans as well as social, cultural, environmental and economic loss of the society [3].

The fight against diseases spread by mosquitoes has enormous environmental, economic and social consequences so that mosquitoes (Diptera: Culicidae) are now referred as 'Public Enemy No. 1' by the World Health Organization in 1996 [7].

According to estimations, mosquitoes transmit the diseases to over 700 million people annually in Africa, South-Central America and much of Asia and cause over 2 million deaths of people each year in the world [8]. There are several important diseases carried by mosquitoes which those are;

### 1.1.1. Malaria

Malaria is the most significant parasitic disease that causes morbidity and mortality in humans. A recent estimate suggests that 91 countries (Figure 1.2) and half of the world's population are at risk of malaria which 225 million cases being recorded in 2009 [9] and

this situation leads to more than 1 million death around world. Most fatal cases are in children under five years of ages [10].

However, most of the deaths (more than 80%) occur in sub Saharan Africa [11]. Although there are many campaigns against disease, malaria is getting worse in Africa because of the resistance of parasites against drugs, mosquitoes against insecticides, the weak health systems and widespread poverty [12, 13].

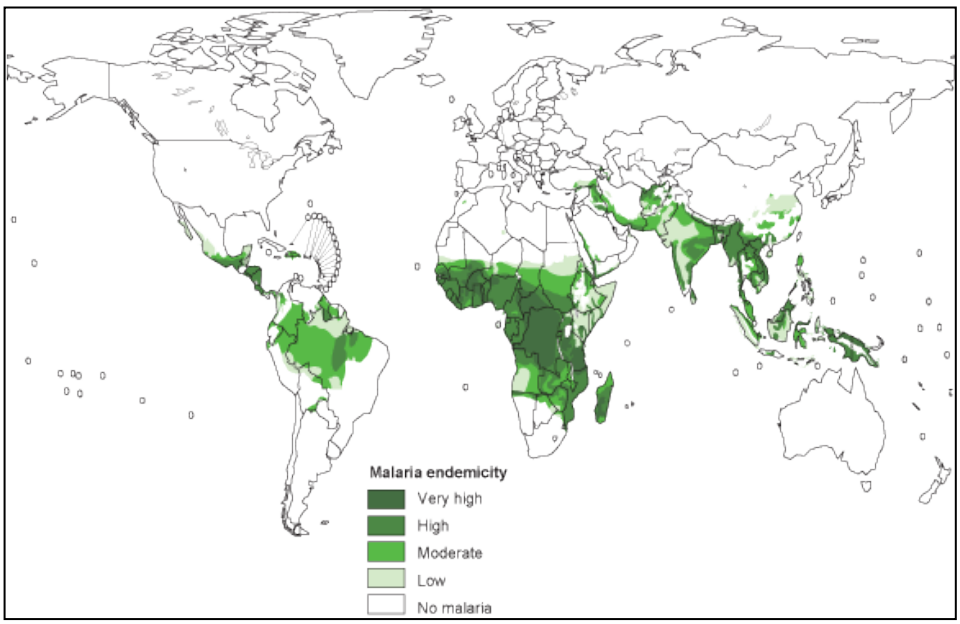


Figure 1.2. Global distribution of malaria transmission risk [14]

Malaria is a mosquito-borne disease of humans and animals caused by protozoans of the genus Plasmodium. The disease is transmitted primarily biting from a female *Anopheles gambiae* [15].

Malaria causes symptoms that typically include fever and headache, which in severe cases can progress to coma or death. The disease is widespread in tropical and subtropical regions in a broad band around the equator, including much of Sub-Saharan Africa, Asia, and the Americas [16].

### 1.1.2. Dengue Fever

Approximately, more than one-third of the world's population living in areas at risk for transmission and this sick is a leading cause of illness and death in the tropics and subtropics [17]. Dengue fever has emerged as a problem around world since 1950s (Figure 1.3) and now it is estimated that 100 million people are infected every year [18]. There is no any vaccine to prevent infection with dengue virus (DENV) so that most effective protection method is that avoid of mosquito bites. When infected, early recognition and prompt supportive treatment can substantially lower the risk of developing this disease [19].

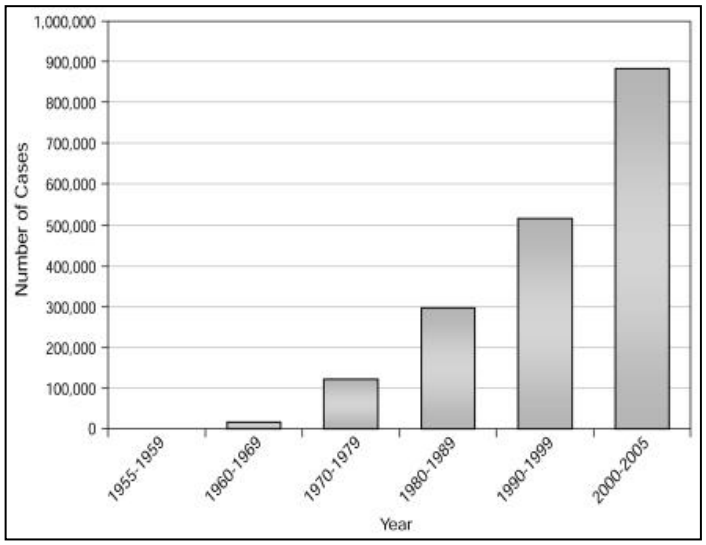


Figure 1.3. Dengue/dengue hemorrhagic fever, average annual number of cases reported to WHO [18]

Primarily, dengue is virus transmitted by *Aedes spp*. mosquitoes, especially *Aedes aegypti*. Other *Aedes* species can also transmit the disease virus such *Aedes polynesiensis*, *Aedes albopictus* and *Aedes scutellaris* [20].

### 1.1.3. West Nile Virus

West Nile Virus (WNV) is an arbovirus and transmitted by a bite of an infected female mosquito [21]. This disease is commonly widespread in Africa, southern Europe, the

Middle East and western Asia. First identification of West Nile virus was occurred in the East African nation of Uganda in 1937 as West Nile sub-region. The first human cases of WNV in Wisconsin appeared in 2002. Few mosquitoes actually carry the virus. This disease was not active until mid-1990s but then the disease has now spread through world dramatically. This case shows us how the mosquitoes are important to fight them [22].

Generally, the disease transmits by *Culex spp.* which differs in different regions such *Culex pipiens, Culex tarsalis* and *Culex quinquefasciatus*. In nature, mosquitoes become infected with WNV by feeding on infected birds and can transmit the virus to other animals, birds, and humans [23].

### 1.1.4. Yellow Fever

Yellow fever (YF) is a viral hemorrhagic fever transmitted by infected mosquitoes. The "yellow" comes from the affects of some patients, causing yellow eyes and yellow skin. Most cases in human are fever, chills, anorexia, nausea, jaundice and headache. This disease is commonly occurring in subtropical and tropical areas such as South America and Africa as well as Asia [24]. According to WHO, there are 200,000 illnesses and 30,000 death people every year around world [25]. An effective vaccine against YF developed since middle of the $20^{th}$ century and some countries require vaccinations for travelers [26].

Yellow fever virus is commonly transmitted by the bite of female *A. aegypti*, but other mosquitoes such as *A. albopictus* can also be a vector of virus. Especially, *A. aegypti* is very well adapted to urban centers so that the mosquito can also cause other diseases, including dengue fever and chikungunya. This situation increases the threat of diseases for cities [27].

## 1.2. MOSQUITOES AND BACTERIA

### 1.2.1. *Bacillus sphaericus*

*Bacillus sphaericus* (BS) is an obligate aerobe and spherical endospore-forming bacterium as well as belongs to *Bacillaceae* family (Table 1.1), a bacterium that ubiquitous in nature.

It is a gram positive bacterium which has rod shaped cells that form chains [28]. *B. sphaericus* mainly used as a larvacide for mosquito control and grows easily on both normal laboratory media as well as on different raw material. Interestingly, there are different strains of *B. sphaericus* that may not have insecticidal activity. Using the classical biochemical identification methods it is not possible to distinguish differences between the insecticidal and non-insecticidal varieties of *B. sphaericus* [29].

Table 1.1. Scientific classification of *B. sphaericus*

| | |
|---|---|
| Kingdom | *Bacteria* |
| Phylum | *Firmicutes* |
| Class | *Bacilli* |
| Order | *Bacillales* |
| Family | *Bacillaceae* |
| Genus | *Bacillus* |
| Species | *B. sphaericus* |

Some strains of *B. sphaericus* synthesize a parasporal inclusion or crystal, which contains proteins toxic for larvae of a variety of mosquito species. During sporulation, the crystal remains associated with the endospore, both being enclosed within the exosporium. The major components of crystal are two proteins which called BinA (42 kDa) and BinB (51 kDa) [30].

The first isolation of a mosquitocidal strain of *B. sphaericus* (strain K) was achieved by Kellen *et al.* [28] from moribund larvae of *Culiseta incidensin* in California. The activity of these strains calculated as so low that they could not be considered for vector control but further studied showed that other strains have toxic affect which the isolation of strains 1593 in Indonesia, 2362 in Nigeria and 2297 in Sri Lanka opened up the possibilities of bacterial mosquito control because of their high toxicity. It showed that these highly toxic isolates all had a similar feature, the synthesis of a crystalline parasporal toxin, that was absent in the earlier, less toxic strains [29, 30]. The crystal of *B. sphaericus* is smaller than that of *B. thuringiensis* crystal protein, but generally visible under a good phase-contrast microscope (Figure 1.4a) [31].

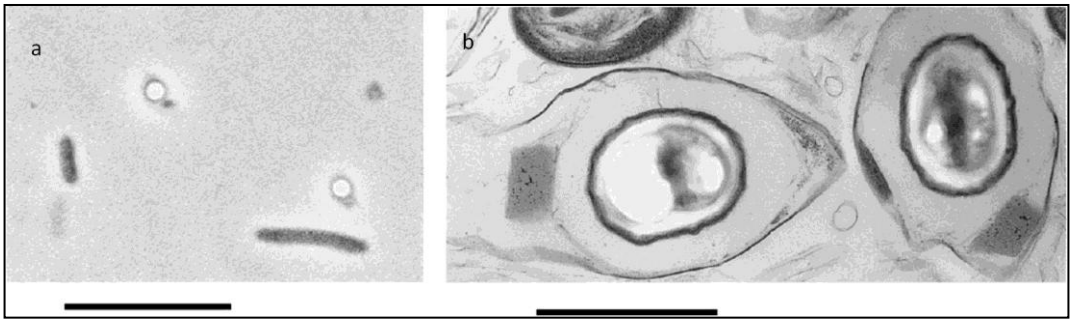


Figure 1.4. Insecticidal crystal proteins (a) Phase-contrast micrograph of *B. sphaericus* strain 9002 and (b) electron micrograph of *B. sphaericus* 2362 showing insecticidal crystal proteins as dark spots associated with phase bright spores and in thin section, respectively. Bars indicate (a) 10μm and (b) 1μm [31]

Over the further 30 years various screening programs resulted in the isolation of mosquitocidal *B. sphaericus* strains from dead insects, soil, mud and water from all around the globe and over 560 strains are now held by the International Entomopathogenic Bacillus Centre at the Institute Pasteur, Paris. There are few of these are more toxic than the original strains 1593, 2362 and 2297 of *B. sphaericus*.

This bacterium affects the larval stages of the mosquito life cycle. Naturally, larvae are ingesting the spore-crystal protein complex as part of its diet. *B. sphaericus* normally cannot negative effect against adult larvae (4. instar). Mosquitoes are varying in their susceptibility, and it is not possible to generalize within genera [32].

*Culex quinquefasciatus* is highly susceptible to *B. sphaericus* ($LC_{50}$=50–100 ng toxin protein/mL) [33] which causes filariasis and Japanese encephalitis by *Culicine* mosquitoes [34, 35].

Some *Anopheline* species (vectors of malaria) are intermediate in their susceptibility (LC50=360– 5000 ng/mL) [33] with *Anopheles albimanus* and *A. quadrimaculatus*. So that *B. sphaericus* are considered to prevent of malaria in several countries [36].

Another mosquito is that *Aedes aegypti* (LC50=42 000 ng/mL) [33] shows intensive effect to *B. sphaericus* but other species of *Aedes* such as *Ae. atropalpusand* and *Ae. nigromaculisare* reasonably susceptible [37].

When ingestion of the spore–toxin complex by a susceptible larva, signs of intoxication start quickly. Ultra structural studies show swelling of mitochondria followed by the appearance of large vacuoles in cells of the gastric caecum and posterior midgut. The midgut cells are messed up and peristalsis ceases. The larvae are expected to dead up to 48 h and the spores germinate, if possible, within the cadaver resulting in the release of some $10^5$ fresh spores per larva [38].

*B. sphaericus* also has no toxic effect against blackflies, has little or no effect on non-target insects, and no effect on mammals. Besides, the effect of *B. sphaericus* can change among mosquitoes larvae in various regions [39-41].

### 1.2.2. The Binary Genes and Toxins of *B. sphaericus*

The genes of crystal toxins of *B. sphaericus* are very highly conserved, especially when compared to the diversity of their *B. thuringiensis*. In *B. thuringiensis*, some toxins have been found against mosquito called *cry* genes. This genes show heterogeneity among *B. thuringiensis* such as cry1A, cry1B, etc and these *cry* genes have also toxic effect other insects than mosquito [42].

Despite intensive screening programmes in search of diversity of insect pathogenicity in *B. sphaericus*, toxicity appears to be restricted to a few mosquito genera and does not extend into the black flies or other biting flies. Four variants of the bin genes have been founded in DNA homology group IIA strains of *B. sphaericus* and in all cases variations in the A gene are matched by changes in the B gene. BinA1 is always related with BinB1 and BinA2 is with BinB2 and so on. (Table 1.2) [43]. In Table 1.2, *bin* gene designations given where known, otherwise presence/absence indicated by +or −. n.d., not determined.

Table 1.2. Some representative strains of DNA homology group IIA *Bacillus sphaericus* and their characteristics [43]

| **Strain** | **Origin** | **Serotype** | ***bin**** |
|---|---|---|---|
| K | USA | 1a | - |
| Q | USA | 1a | - |
| 9002 | India | 1a | A1B1 |
| SSII-1 | India | 2a2b | - |
| 1883 | Israel | 2a2b | - |
| LP24-4 | Singapore | 2a2b | - |
| LP35-6 | Singapore | 2a2b | - |
| BDG2 | France | 3 | - |
| IAB 881 | Ghana | 3 | A1B1 |
| LP1-G | Singapore | 3 | A4B4 |
| LP7-A | Singapore | 3 | A4B4 |
| 1593 | India | 5a5b | A2B2 |
| 2362 | Nigeria | 5a5b | A2B2 |
| BSE 18 | Scotland | 5a5b | A2B2 |
| IAB 59 | Ghana | 6 | A1B1 |
| IAB 481 | Ghana | 6 | + |
| IAB 774 | Ghana | 6 | + |
| R-1e | Brazil | 6 | - |
| Gt-1a | Brazil | 6 | - |
| 2297 | Sri Lanka | 25 | A3B3 |
| M2-1 | Malaysia | 25 | + |
| 2377 | India | 26a26b | - |
| 2315 | Thailand | 26a26b | - |
| IMR 66.1S | Malaysia | 48 | - |
| Pr-1 | Scotland | n.d. | A1B1 |
| IAB 872 | Ghana | 48 | A1B1 |

The differences of *bin* genes are little. The BinA1 gene differs from BinA2 in only two bases resulting in the glutamate residue at position 104 of BinA1 being replaced by a serine residue in BinA2. For BinB genes, the situation is different which in five amino acid changes between BinB1 and BinB2. Similarly, BinA3 and BinA4 are highly related, differing by only two amino acids at positions 93 and 99. It is detected that the differences of bin genes result with different toxicity. For instance, the BinA4B4 proteins from strain LP1-G confer on this strain a lower toxicity against *C. quinqefasciatus* than the other variants [43-45].

To increase the toxicity of *bin* genes, some researches were studied such as site-directed mutagenesis. It revealed that alterations at residues 99 and 104 of BinA were sufficient to account for these changes in toxicity. This is close to the unique ser-93 of BinA4 and together probably identifies the active site of the BinA toxin in the region 93 to 104 [37, 46].

As toxin proteins of *B. sphaericus*, the major toxin of *B. sphaericus* comprises the proteins of parasporal crystal. This crystal accumulates within the exosporium (Figure 1.4b). The crystal is composed of two polypeptides with the molecular weights of 42 kDa (BinA) and 51 kDa (BinB) and they together called binary (Bin) toxin. Generally, all strains which have high toxicity contain the Bin toxin. Upon ingestion of toxins by mosquito larvae, these crystal proteins dissolve in the high pH of the insect midgut. BinA is slowly converted into a smaller protein of around 39 kDa by insect proteinases and BinB is more rapidly processed to an active form of around 43 kDa [47, 48]. Both proteins are required for optimal toxicity. Early studies were confused on this point because of the difficulties of separating the two proteins biochemically, but once individual proteins were prepared from cloned genes in *Escherichia coli*, it became apparent that BinAB comprised a true binary toxin similar in context to the diphtheria and cholera toxins [49, 50]. However, it showed that BinA alone will lyse cultured cells of *C. quinquefasciatus* [29] and in large amount of this protein will kill mosquito larvae [51], but the BinB protein alone, even in large excess, is not toxic [52, 53].

The mechanism of toxin proteins is that the binary toxin complex binds to midgut of cells of susceptible larvae through the BinB component and it allows internalization of BinA

[54]. There is a strong and regionalized binding of the complex and BinB shows the same regional binding as all binary toxin, whereas BinA shows only weak binding throughout the midgut. However, in the presence of BinB, BinA toxin becomes stronger and this means that BinB affects the specific binding. [55].

The biochemistry of the BinA is not known well, but it is estimated that it may involve pore formation in the midgut cells because in vitro assays in planar lipid bilayers and permeabilization measurements in liposomes have confirmed channel formation by BinA [37].

As to BinB, it binds in *C. quinquefasciatus* of a specific receptor [56] and BinB is susceptible against *Anopheles* larvae , however, not to the resistant larvae of *Ae. aegypti*. From this view, the target range is determined by the availability of receptor sites rather than by proteolytic processing in the midgut of larvae. The receptor in *Culex pipiens* has now been identified as an alpha-glucosidase of around 60 kDa that is localized to the membrane of the cells by a glycosyl-phosphatidylinositol anchor. In *Anopheles gambiae*, the binding protein has not been fully identified, but the candidate site is also attached to the cell surface by a glycolipid anchor [57, 58].

### 1.2.3. Mosquitocidal (Mtx) toxins

Some *B. sphaericus* strains, such as strain SSII-1, have toxic affect against mosquito despite lack of a parasporal crystal. The reason of this situation is Mtx gene or mosquitocidal toxin. Three different types are known for Mtx specifically – Mtx1 (100 kDa), Mtx2 (31 kDa), and Mtx3 (36 kDa). Most important is Mtx1 which the protein of around 100 kDa accumulates in vegetative cells and, when ingested by larvae, is cleaved into two subunits of 27 and 70 kDa which resemble an ADP ribosylating toxin and a glycoprotein-binding protein, respectively [59, 60]. Normally, Mtx1 has highly toxic activity to mosquito larvae (LC50=15 ng/mL), especially for *Ae. Aegypti* [61], but as a bacterium its overall toxicity is low because of low gene expression [62] and proteolytic degradation in the vegetative cell [63]. It can be a good toxin with enhancing of gene expression in a reduced proteolytic background for biocontrol purposes.

### 1.2.4. Mosquitoes

Mosquitoes are a family of the small, nematocerid flies and they belong to Culicidae family (Table 1.3). Most common known mosquitoes genus are *Aedes*, *Anopheles* and *Culex*. Even if only a few species are harmless, most of them are considered harmful for humans [64].

Table 1.3. Scientific classification of mosquitoe

| | |
|---|---|
| Kingdom | Animalia |
| Phylum | Arthropoda |
| Class | Insecta |
| Order | Diptera |
| Suborder | Nematocera |
| Infraorder | Culicomorpha |
| Superfamily | Culicoidea |
| Family | Culicidae |

Mosquitoes are insects that have been around for more than 30 million years. It seems that, during those millions of years, mosquitoes have been hiding their skills so that they are now experts at finding people to bite. There are over 3500 different species of mosquitoes throughout the world. Mosquitoes transmit pathogens that cause diseases known as malaria, yellow fever, dengue fever etc. On the other hand, they sometimes carry different diseases from one person to another during feeding [65].

Although their harmfulness is accurate, they have positive effect in nature such as being a food for fish and other predatory aquatic animals during larvae stage. Adult mosquitoes are also important food for birds, bats and other arthropods including dragonflies and spiders. All mosquitoes must live in water to complete their life cycle. The quality of water can change from melted snow water to sewage effluent and it can be in any container imaginable. The length of the life of adult mosquito depends on several factors: sex, temperature, humidity and time of year. Genetically, most males live a very short time,

about one week, and the females live about a month which depends on the factor listed above. With all this information, the diseases are carried by female mosquitoes [66].
The life span of mosquito goes through four separate and distinct stages such egg, larva, pupa and adult or imago (Figure 1.5). Each of these stages can distinguish by their special appearance.

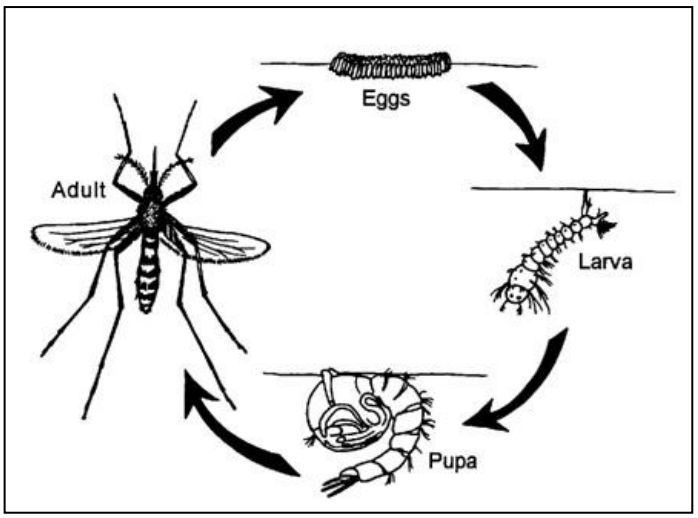


Figure 1.5. Life cycle of mosquito (http://www.wwhd.org/mosquito_facts.htm)

Eggs of mosquito are laid one at a time and they float on the surface of the water that they live. However, the eggs of *Culex* and *Culiseta* species are stuck together in rafts of a hundred or more. *Anopheles* and *Aedes* species do not make egg rafts but lay their singly. Females of many species can lay 100-200 eggs during adult stage of their life cycle. Most eggs hatch into larvae within 48 hours [67].

Larva stages are most important during life cycle of mosquito. This stage also occurs in the water and the larva comes to surface to breathe. Larvae shed their skin four times to become larger in this stage. Most larvae breathe through spiracles located on their eighth abdominal segments so that they must come to the surface of water (Figure 1.6). Some larvae such as *Anopheles* do not have spiracles so that they have to lay parallel to the water surface. There are four larva stage called instar and second-third instar of larva is useful for toxicity assay. During larva stage, it feeds with mostly algae and bacteria and other organic

matter in water. For this reason, larva stage is quite important to control mosquitoes for researchers [68].

Figure 1.6. A group of *Culex* larvae in standing water [69]

The pupa stage can call a resting and non-feeding stage. The mosquito pupa is comma-shaped and the head and thorax are merged into acephalothorax, with the abdomen curving around underneath. The pupa can swim actively by flipping its abdomen, and it is commonly called a "tumbler" because of its swimming action. This stage is a gate to turn to mosquito into an adult. It takes about two days. When the pupa stage is complete, the pupal skin cracks and the mosquito emerges as adult [70].

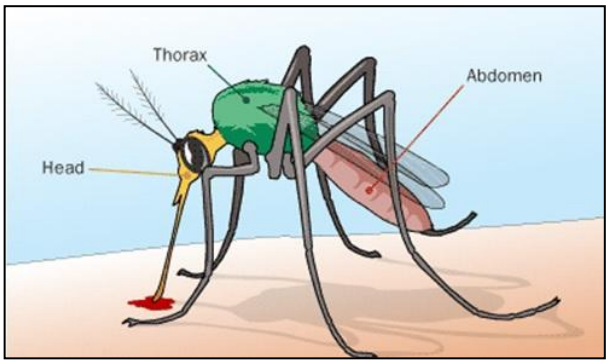


Figure 1.7. An illustration of adult mosquito
(http://mosquitosquadblog.files.wordpress.com/2009/07/mosquito-parts1.gif?w=620)

Last period of development from egg to adult is called as adult stage (Figure 1.7) which all diseases are carried in this stage. Some species of mosquitoes can develop to adult from egg in five days but this period can change according to environment conditions. Only female adults bite animals, humans and drink blood that means they causes diseases, but males don't bite which they generally feed nectars of flowers [71].

Turkey is most appropriate place for mosquitoes due to having suitable climate and ecology. In 2001, Ramsdale *et al.*[72] have been reported that there are 8 different genus and 48 different species of mosquitoes in Turkey. According to study, most common and dominant mosquito genus is *Ochlerota* genus (formerly known *Aedes*) with 15 species (*Ochlerota echinus, O. geniculatus, O. caspius, O. communis, O. detritus, O. dorsalis, O. pulchritarsis, O. phoniciae, O. nigrocanus, O. excrucians, O. flavescens, O. zammitii, O. lepidonotus, O. refiki, O. rusticus*) and flowing *Culex* genus with 13 species (*Culex modestus, C. pusillus, C. laticinctus, C. mimeticus, C. perexiguus, C. pipiens, C. theileri, C. torrentium, C. tritaeniorhynchus, C. deserticola, C. hortensis, C. martini and C. territans*).

Other genus is *Anopheles* with 10 species (*Anopheles algeriensis, An. claviger, An. hyreanuss, An. maculipennis, An. marteri, An. plumbeus, An. sacharovi, An. subalpinus, An. pulcherrimus and An.superpictus*). *Aedes* is with 3 species (*Aedes einereus, A. vexans and A. eretinus*).

Table 1.4. The mosquitoes species and their breeding areas in Antalya, Turkey [73]

| | **Breeding Areas** | | | | | | |
|---|---|---|---|---|---|---|---|
| **Mosquito species** | **Cesspool** | **Basement** | **Brook** | **Cliff** | **Swamp** | **Pool** | **Barrel** |
| ***C. pipiens*** | * | * | * | | * | * | * |
| ***C. martini*** | | | | | | * | |
| ***C. deserticola*** | | * | * | | | | * |
| ***O. caspiu*** | | | | * | | * | |
| ***An.superpictus*** | | | * | | | | |
| ***C. longiareolala*** | * | * | | | * | | |

*Culiseta* genus is with 4 species (*Culiseta longiareolala, C. fumipennis, C. morsitans and C. annulata*). *Mansonia* is with *Mansonia richiardii*, *Orthopodomyia* is with *Orthopodomyia pulcripalpis* and, lastly, *Uranotaenia* genus is with *Uranotaenia unguiculata* species.

These species generally live in different places, especially the places where have pollutant places. In a study, Çetin *et. all.* [73] showed that the places where the mosquitoes breed mostly in Antalya, Turkey (Table 1.4).

# 2. MATERIALS

## 2.1. INSTRUMENTS

The instruments used in this study are as follows:

- Laminar Flow Cabinet (Heal Force, Hfsafe-1200, Class II Biological Safety Cabinet, USA)
- Centrifuge (SIGMA 1-14 centrifuge, Germany)
- Incubator (Binder, USA)
- Vortex (Stuart SA8, UK)
- Protein Electrophoresis System (Bio-Rad, USA)
- Agarose Gel Electrophoresis System (Elite 300 Plus, Wealtech, USA)
- Incubator Shaker (New Brunswick Products, USA or Sartorius, Germany)
- pH meter (Hanna instruments PH211, Germany)
- Heater (Heidolph, Germany)
- Spectrophotometer (Pharmacia Biotech, USA)
- Sub Aqua 26 Plus Water Bath (Grant, UK)
- Autoclave (Tutnauer, Netherlands)
- Sonicator (WiseClean, Thailand)
- PCR Thermal Cycler (Bio-Rad, USA)
- Nanodrop (2000, Thermo Scientific, USA)
- Molecular Imager (ChemiDoc XRS+ and Gel Doc XR+, Bio-Rad, USA)
- Microbial Identification System (Newark, Germany)
- -80 °C freezer (Sanyo, USA)
- ELISA plate reader (Bio-Tek EL x 800, USA)
- Biolog microplate reader (BIOLOG, USA)
- Rotary Evaporator (Buchi, Italy)

## 2.2. EQUIPMENTS

The laboratory equipments used in this study are as follows:

- Serological pipettes 25, 10, 5, 2 ml (Grenier-Bio or Axygen, USA)
- Polypropylene centrifuge tubes, 50 ml, 15 ml, 2 ml, 1 ml, 0.5 ml (Isolab, Germany)
- Micro pipettes 1000, 200, 100, 10, 2.5 µl (Thermo Scientific, USA)
- Inoculation Loops (Isolab, Germany)
- Pasteur Pipette (Isolab, Germany)
- Petri Plate (Isolab, Germany)
- Erlenmayer, Bottles, Falcons (15,50 ml) (Isolab, Germany)
- Cryotubes (TPP, Switzerland)

## 2.3. CHEMICALS

- Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Fermantas, R0392, Germany)
- Ampicillin Sodium Salt (Sigma, A9518, USA)
- 0,5-10 kb DNA Ladder Marker (Bio Basic Inc., 070919, Canada)
- SiZer™-1000 plus DNA Marker (Intron Biotech., 24075, Korea)
- PageRuler Prestained Protein Ladder (Thermo Scientific, 26616, Germany)
- TALON Superflow Metal Afinity Resin (Takara Bio Inc., 635507, Japan)
- cOmplete ULTRA Tablets, Mini, *EASYpack* (Roche, USA)
- Dithiothreitol (DTT) (Fisher Scientific, BP172-5, USA)
- Biolog Universal Growth Agar + %0.025 Maltose (BUG+M) (BIOLOG, USA)
- Imidazole (Sigma, 15513, USA)
- Albumin From Bovine Serum (Sigma, A7906, USA)
- Nutrient Broth (Merck, 105443, Germany)
- Tryptone Soya Agar (Oxoid, CM0131, UK)
- Tris Acetate-EDTA Buffer (Sigma, T6025, USA)
- Glycerol Solution (Sigma, 15524, USA)
- Agarose (Sigma, A9539, USA)
- Sodium Phosphate Dibasic (Sigma, 04272, USA)

- Trypsin-EDTA (Biochrom L2153, Germany)
- Absolute Ethanol (Sigma, 32221, Germany)
- 2-propanol (AppliChem A3928, Germany)
- Ethylenediaminetetraacetic acid (EDTA) (Fisher Scientific, BP1302-10, USA)
- NdeI Restriction Enzyme (New England Biolabs Inc., R0111S, UK)
- BamHI-HF Restriction Enzyme (New England Biolabs Inc., R3136S, UK)
- T4 DNA Ligase (New England Biolabs Inc., M0202S, UK)

## 2.4. KITS

- Genomic DNA Isolation Kit (Invitrogen, K182001, USA)
- Plasmid Isolation Kit (Invitrogen, K210005, USA)
- Ready %4-12 Bis-Tris SDS Gel (Novex, NP0321, Canada)
- Phusion DNA Polymerase Kit (Finnzymes, F-530, Finland)
- Sigma GenElute PCR Clean-Up Kit (Sigma, NA1020, USA)
- Invitrogen PureLink Quick Gel Extraction Kit (Invitrogen, K2100, USA)

# 3. METHODS

## 3.1. BACTERIAL CULTURES

*B. sphaericus strain* (MBI5) was obtained from the culture collection of Microbiology Laboratory, Department of Genetics and Bioengineering, Yeditepe University. Bti 4Q4, Bti ATCC 35646, a *B. sphaericus* were used as reference bacteria during bioassay tests. *E. coli* DH5α and *E. coli* BL21 were used during cloning process of toxin genes. These strains were provided from the culture collection of Plant Biotechnology Laboratory, Department of Genetics and Bioengineering, Yeditepe University.

## 3.2. ISOLATION AND IDENTIFICATION OF BACTERIA FROM MOSQUITO LARVAE

Mosquito's larvae collected from natural habitat around Istanbul, Turkey. Unhealthy larvae samples were selected and used for isolation of bacteria. 42 unhealty larvae were used in this study. Each larva crashed from midgut area and plated onto nutrient agar (NA) medium for isolation of the microorganisms. All plates incubated at 30 $^{o}$C for 2 days. After 2 days incubation period, all individual bacteria selected and purified on NA. Totally 252 bacterial strains were isolated from larvae and stored in 15% glycerol at -80°C to be used in further tests.

Carbon Substrate Utilization (BIOLOG):

Bacterial strains were tested for carbon substrate utilization by using Biolog Microplate (GP) system. A pure culture was isolated from BUG + M agar. When the culture was pure, it was subcultured on BUG + M + T (0.25% maltose swabbed with thioglycolate). Then thioglycolate was added to the agar plate: before streaking the strain, precisely 8 drops from a thioglycolate dropper was added into 3 ml of sterile water. A sterile swab was dipped into the solution to moisten the cotton tip. A thin film of liquid was spreaded across the entire surface of the agar medium. For the thioglycolate to dry on the agar, it was allowed for approximately 5 minutes. Secondly Biolog's sterile stick was used to touch a

colony and make a plus sign (+) on the center of the agar media going across each of the two lines and media were incubated at 27°C for 24h. Cells were harvested with sterilized plastic loops, and suspended in sterile saline (0.85 NaCl pH: 6). The Biolog GP Microplates were preconditioned at 27°C for 24h, then inoculated by adding 150 ml of each bacterial suspension into the reaction wells of microplate using a multichannel micropipetter. The plates were incubated at 27°C for 12-24h. The color reaction inditory positive utilization of each carbon substrate was read by Microplate reader and the results (metabolic fingerprint) for each bacterial strain were compared with the Biology GP database with Microlog Software (v 4.20.03).

FAME Analysis:

Extraction and identification of whole-cell fatty acid methyl-esters (FAME's) by gas chromatography was performed. Bacteria were streaked onto TSA and incubated at 80°C for 24h. A loopfull of cells were harvested from TSA plate of each strain and added to 1,2 M NaOH in 50% aqueous methanol in a screw cap tube, then incubated at 100°C for 30 min in water bath. After, saponified samples were cooled at room temperature for 25 min. They were acidified and methylated by adding 2 ml 54% 6 N HCL in 46% aqueous methanol and incubated at 80°C for 10 min in water bath. After rapid cooling, methylated fatty acids were extracted with 1, 25 ml 50% methyltert butyl ether (MTBE) in hexane. Each sample was mixed for 10 min and bottom phase was removed with a Pasteur pipet. The top phase was washed with 3 ml 0,3 M NaOH. After mixing 5 min, the top phase removed for analysis. Fatty acid methyl esters were separated by gas chromatography FAME profiles of the strains were identified by the commercial TSB6 database with Microbial Identification System software (v 6:0). The cellular concentrations of the fatty acids for each strain were determined and strains were identified at species level.

16S rRNA Analysis:

16S rRNA genes of the bacterial DNA isolates (MBI5) were amplified by the PCR using purified DNA and primers 27f and 1492r. PCR amplifications was caried out in total volume of 50 µl reaction mixture containing 0.2 mM of 27f and 1492r primers for total 16S, 0,2 µl of DNA polymerase, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 1

mM MgSO4, 10mM Tris and 50 ng template DNA. PCR conditions were as follows : preamplification 94°C for 5 min : denaturation at 94°C for 30s : annealing at 55°C for 40s : elongation at 72°C for 2 min repeated 35 cycles and then post amplification for final extension 10 min at 72°C.

### 3.3. BIOASSAY TEST OF BACTERIA

Single colonies of newly isolated bacterial strain (MBI5) and Bti 4Q4, Bti ATCC 35646, a reference *B. sphaericus* were cultivated on NA and incubated for 48h at 30°C. Bacterial growth of each strain was harvested and resuspended in 10 ml of distilled water. Absorbance was adjusted to 0.2 with water and then 1 ml of suspension was added to 100 ml of fresh water/polluted water in 250ml flasks containing 100 larvae (at the stage of $2^{nd}$ or $3^{rd}$ instar) of *Culex* spp. The inoculated flasks were maintained on laboratory bench and observed for 48h at room temperature. In order to determine larvicidal bacterial strains, which were capable of killing 90% of larvae, positive and negative control flasks treated with reference strains and sterile water, respectively, were kept the same condition as inoculating ones. After toxicity test, *B.sphaericus* (MBI5) was selected as high toxic mosquitocidal strain and used for further studies.

### 3.4. ISOLATION OF GENOMIC DNA AND PLASMID

Bacterial genomic DNA was isolated using Invitrogen PureLink Genomic DNA Mini Kit and plasmid was isolated using Invitrogen PureLink HiPure Plasmid Midiprep Kit according to the manufacture instructions.

### 3.5. POLYMERASE CHAIN REACTION (PCR) OF GENES

To amplify the BinA and BinB genes from the genome and bacteria (colony PCR), Finnzymes High-Fidelity DNA Polymerase kit was used. The PCR amplification was carried out in eppendorf thermal cycler in 20 µl reaction volume. The reaction was subjected for amplification from genomic DNA to initial denaturation of 2 min at 95°C and subsequent 35 cycles each comprising denaturation of 92°C for 50 s, annealing at 55°C for 50 s and elongation at 72°C for 50 s. For colony PCR, the reaction was subjected to initial

denaturation of 7 min at 95°C and subsequent 35 cycles each denaturation of 92°C for 50 s, annealing at 55°C for 50 s and elongation at 72°C for 50 s. The pipetting instruction listed below (Table 3.1).

Table 3.1. PCR Pipetting Instructions

| **Component** | **Volume / 20 µl reaction** |
|---|---|
| $H_2O$ | 11,4 µl |
| 5x Fhusion HF Buffer | 4 µl |
| 10 mM dNTPs | 0,4 µl |
| 10 mM Forward Primer | 0,5 µl |
| 10 mM Forward Primer | 0,5 µl |
| DNA | 3 µl |
| DNA Polymerase | 0,2 µl |

## 3.6. PREPARATION OF COMPONENT CELL

E. coli DH5α (BL21) was used to prepare a component cell.

Method

1. 10 ml LB was inoculated with *E. coli* and incubated at 37°C with 180 rpm overnight.
2. After incubation, 1 ml of inoculum was added into 35 ml LB and incubated at 37°C with 180 rpm for 3 h.
3. The mix was centrifuged at 5500 rpm for 10 min and supernatant was discarded.
4. 5 ml of fresh and cold $CaCl_2$ (100 mM) was added very slowly and incubated for 1 h on ice.
5. After 1 h, the mix was centrifuged at 3500 rpm for 5 min.
6. 1 ml of supernatant was taken and rest of supernatant was discarded. Taken 1 ml supernatant was dissolved with pellet.
7. Component cells were allocated and saved at 4°C for 7 days.

## 3.7. AGAROSE GEL ELECTROPHORESIS

1% agarose gel was prepared with the buffer, Tris Acetate- EDTA buffer (Sigma). The gel preparation protocol is given in detail below;

- A 1% agarose gel was made by mixing 0,5 g agarose with 50 ml TAE buffer.
- The mixture was heated in a microwave oven until all agarose had melted and the solution had started to boil.
- It was waited to get a cool solution approx. 60-65 °C.
- 2,5 µl ethidium bromide was added into solution and gently mixed.
- The gel was poured into gel tray and the comb was set. All bubbles were removed.
- After 20 min, when the gel had solidified, the tray was released from all components.
- The tray was set into tank containing TAE and samples were loaded with marker.
- The gel was run at 80 V for 40 min. Finally, gel was visualised under UV light.

## 3.8. BRADFORD PROTEIN ASSAY

Bradford assay allows determining the concentration of proteins in solution. 2 mg/ml Bovin Serum Albumin (BSA) was prepared with $dH_2O$. Seven standard protein solutions in different concentration was prepared 125, 250, 500, 750, 1000, 1500 and 2000 µg/ml of 2 mg/ml BSA. These protein concentrations were used to drawn a standard curve (Figure 3.1).

Preparation Procedure

1. 5 µl of sample was added into 250 µl Sigma Bradford Reagent in a 96-well plate.
2. The solution was incubated for 5 min and protected from light.
3. Finally, the absorbance was measured at 590 nm with spectrophotometer.

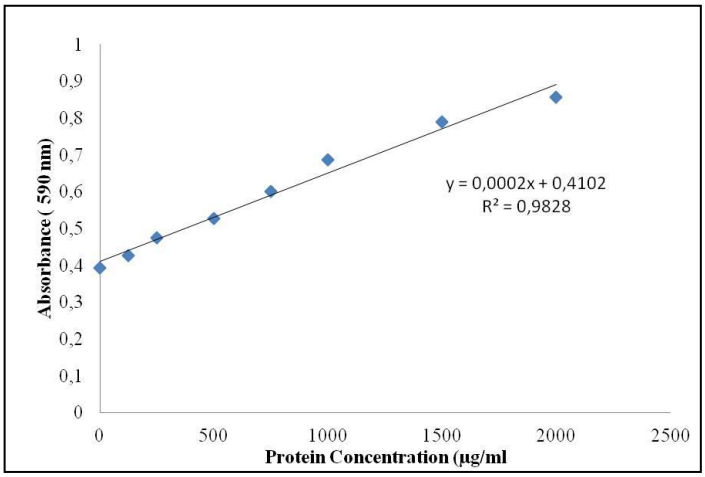


Figure 3.1. Standard curve of Bradford Protein Assay

## 3.9. CLONING AND EXPRESSION OF BINA AND BINB GENES

The primers specific for BinA and BinB genes were designed based on the sequences available in GenBank (accession number AJ224477). The forward and reverse primers for BinA and BinB genes were stated in Table 3.2.

Table 3.2. Designed primers for BinA and BinB genes

| | | |
|---|---|---|
| **BinA** | Forward | 5'-GCTTAACATATGAGAAATTTGGATTTTATTG-3' |
| | Reverse | 5'-ATGTGGATCCTTAGTTTTGATCATCTGTAATAATC-3' |
| **BinB** | Forward | 5'-ACAGCATATGTGCGATTCAAAAGACAATTCTGGCG-3' |
| | Reverse | 5'-AGACGGATCCTCACTGGTTAATTTTAGGTATTAATTC-3' |
| *The engineered restriction sites NdeI and BamHI are underlined, respectively | | |

The primers designed with NdeI and BamHI restriction sides. As the plasmid (pET-16b) used in this study includes these two enzyme sequence at the multiple cloning side (MCS) (Figure 3.2). The plasmid has also N-terminal His-Tag sequence as well as having ampicillin sequence as a selection marker. His-Tag allows isolating target proteins using affinity feature with protein isolation kit or resin.

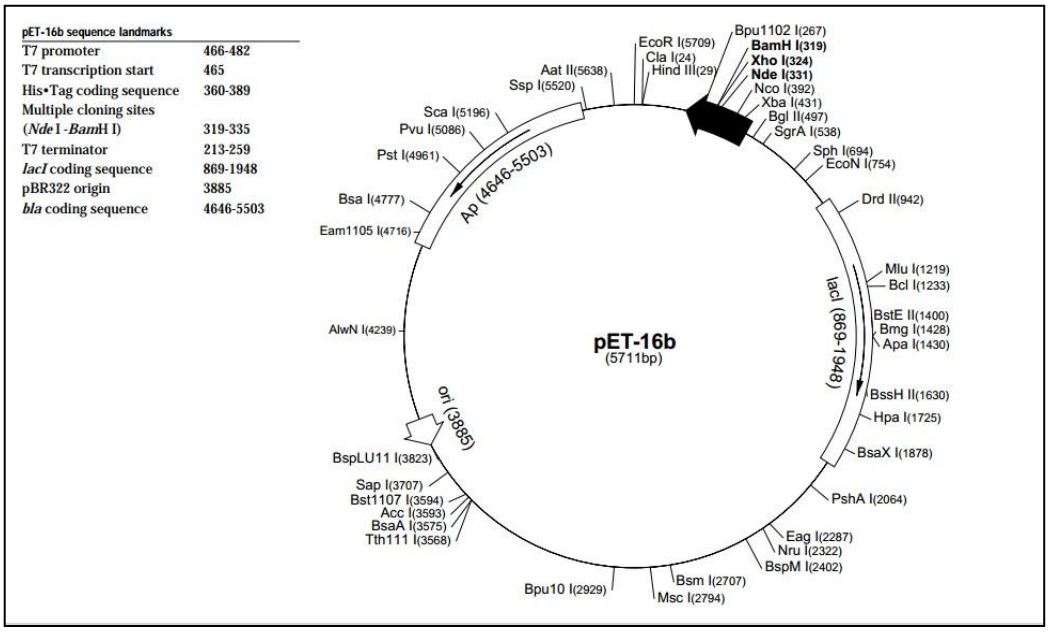


Figure 3.2. Sequencing map of pET-16b plasmid

The BinA and BinB genes were amplified by PCR and the plasmid was digested with NdeI and BamHI restriction enzymes. Then, amplified genes were digested using NdeI and BamHI restriction enzymes (Table 3). PCR products of these genes were cleaned up by Sigma GenElute PCR Clean-Up Kit and plasmid was purified by using Invitrogen PureLink Quick Gel Extraction Kit after running on an agarose gel according to the manufacturer recommendations. PCR products (inserts) and digested plasmid (vector) were incubated at 16°C overnight with a molar ratio of 1:3 vector to insert, respectively. NEB T4 DNA Ligase Ligation protocole was used in ligation process..

Table 3.3. Pipetting Instructions of Restriction Enzymes

| **Component** | **Volume** |
|---|---|
| Restriction Enzyme | 0,5 µl (20,000 units/ml) |
| DNA | 1 µg |
| 10x Restriction Enzyme Buffer | 5 ml |
| Total reaction volume ($dH_2O$) | 50 µl |
| *Incubated at 37 °C for 1 h | |

The recombinant plasmid was subsequently transformed into *E.coli* DH5α by heat shock transformation method. Briefly, 2 µl recombinant plasmid was inoculated with 50 µl component cell for 15 min on ice in a test tube. Subsequently, the tube was incubated at 42°C for 90 sec and then 2 min on ice immediately. The mix was incubated with 200 µl Luria Broth (LB) at 37°C for 1,5 h with 200 rpm. Lastly, component cells were inoculated onto LB supplemented with 50 µg ml $^{-1}$ ampicillin and waited for 18 h at 37°C.

As the *E. coli* (DH5α) colonies were grown, the colonies were checked where they have insert into plasmid by colony PCR method. Then, the construct plasmid was isolated and transformed into *E. coli* BL21 by heat shock transformation method.

The recombinant BinA and BinB proteins were expressed and purified from *E. coli* BL21 cells. The *E. coli* cells containing pET16-BinA (pET16-BinB) construct were grown at 37°C and 180 rpm in 10 ml LB medium containing 50 µg ml$^{-1}$ ampicillin for overnight. 2 ml broth was inoculated into 100 ml LB medium containing ampicillin. The cells were induced at the late log phase ($A^{600}$~0.7) by adding of 0,4 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 20°C, 120 rpm and bacteria were collected 4 h later. Bacteria were centrifuged at 13.000 rpm and the pellet was boiled by 200 µl 1xSDS dye at 95°C for 10 min. The total proteins from induced cultures were resolved on 12% SDS–PAGE.

## 3.10. SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROHORESIS (SDS-PAGE)

Both induced bacteria and purified toxin proteins were confirmed by SDS-PAGE. The samples were prepared as following;

- 1xSDS dye was prepared containing 30 µl ml$^{-1}$ β-mercaptoethanol
- 200 µl 1xSDS dye for bacteria sample and 20 µl 1xSDS dye for purified protein sample were used. For protein sample 1:1 (SDS:Protein) ratio was used.
- The samples were boiled at 95 °C for 10 min.
- 20 µl samples with 5 µl marker were loaded onto %12 polyacrylamide gel and run at 90 V for 30 min following 120 V for 2 h.

## 3.11. COMASSIE BLUE STAINING METHOD

The SDS-PAGE gel has to stain to observe protein bands. At the end of SDS-PAGE analysis, the gel was treated with Comassie Blue (50% Methanole, 10% Glacial Acetic Acid and 40% $H_2O$) for 30 min with moderate shaking. Gel stained with comassie blue was heated at microwave to increase the efficiency of stain. The gel then was washed with $dH_2O$ twice for 30 min and overnight lastly. The Whatman paper slices were added of gel during washing step to absorb excessive dye. The water was removed and gel was ready for visualization.

## 3.12. PURIFICATION OF THE BINA AND BINB PROTEIN

Purification of BinA and BinB proteins from total protein was performed using TALON® Superflow Metal Affinity Resin. The process was continued until centrifugation period as described at Section 1.5. To recover proteins as in soluble form, the pellet was incubated with lysis buffer (50 mM Tris–HCl pH 8.0, 15% Sucrose, 2 mM dithiothreitol and 1 tablet of Roche Complete® protease inhibitor cocktail and lysozyme to a final concentration of 2 mg $ml^{-1}$) for 1,5 h at room temperature. Cell suspension was further sonicated in pulse mode (7 pulses of 15 s each) and centrifuged at 21,000 g at 4°C for 30 min. Supernatant was collected.

The column was loaded with 2 ml resin and 30 ml equilibration/wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, total pH 8.0). The supernatant was also loaded onto column. The column was washed with 30 ml equilibration/wash buffer. Finally, imidazole elution was performed, in which 10 ml elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 150 mM imidazole, total pH 8.0) was used to eluted protein. The eluted protein was dialyzed overnight at 4 °C against 50 mM sodium phosphate, 300 mM sodium chloride (pH 8.0). The eluted proteins were resolved on 12% SDS–PAGE.

## 3.13. TOXICITY ASSAYS OF BINARY TOXINS

Three different experimental set up were designed. BinA, BinB and BinA+BinB were investigated for their toxicity against susceptible third instar larvae of mosquito *Culex pipiens*. The larvae culture was obtained from Faculty of Biology, Akdeniz University (Antalya, Turkey). Sole proteins and combination of proteins were tested in 30 ml water containing 10 third instar larvae with three replications. The total larval mortality was scored for 6., 7., 8., 9., 10., 12., 24. and 48. h of treatment. Mortality data was analyzed by using EPA Probit Analysis Program (v.1.5) and the $LC_{50}$ values were calculated at 95% confidential limit.

# 4. RESULTS

## 4.1. ISOLATION AND IDENTIFICATION OF BACTERIA

Mosquito's larvae collected from Istanbul around summer season by collaboration with Istanbul Metropolitan Municipality. 252 bacterial strains were isolated from 42 unhealty larvae. All of the isolated bacteria strains were tested for mosquitocidal activity (Figure 4.1).

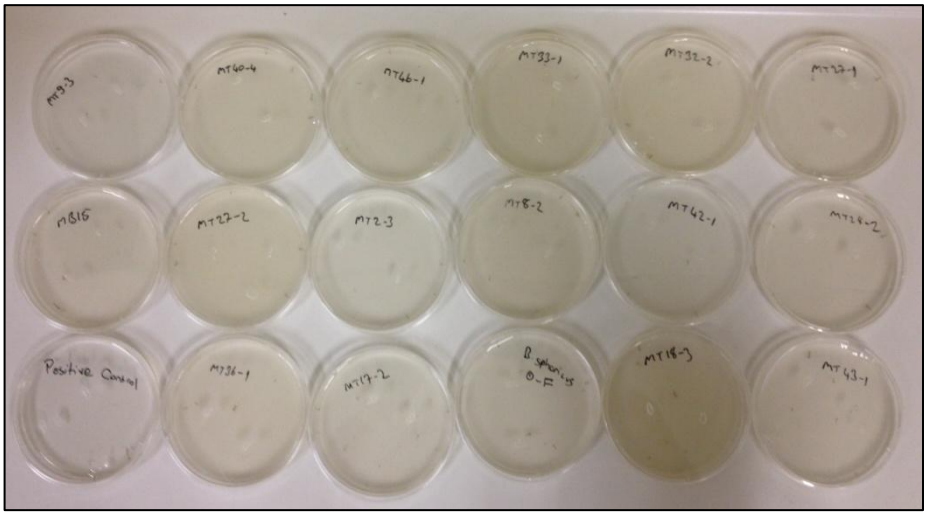


Figure 4.1. Toxicity assay of isolated bacteria from dead or sick larvae on healthy larvae

None of the newly isolated strains in the present study were found to be larvacidal against 3rd instar of *Culex pipiens* tested. The strain (MBI5) which obtained from the culture collection of Microbiology Laboratory, Dept. Genetics and Bioengineering, Yeditepe University was used for further studies. To identify the strain, FAME Analysis (Table 4.1), BIOLOG (Figure 4.2) and 16S-rRNA sequencing analysis (Figure 4.3) were performed.

When compared fatty acid profiles of MBI5 and reference BS, The major cellular fatty acids in MBI5 included iso-pentadecanoic acid ($C_{15:0}$ iso, 45,00%) and $C_{16:0}$ iso, 12,65% and minor amounts of the iso-branched fatty acids $C_{14:0}$ iso (0.60%), $C_{16:0}$ (1.72%), $C_{17:1}$

iso ω10c (1,43%). In contrast, the reference *B. sphaericus* has different amounts of fatty acids. Besides, another fatty acid (14:0 iso 3OH) only appears at reference *B. sphaericus*. Consequently, significant similarities in fatty acids profiles were found between *B.sphaericus* and MBI5. Both MBI5 and *B.sphaericus* were identified with MIDI as *Bacillus-sphaericus*- GC subgroup E.

Table 4.1. Comparison of MBI5 fatty acid profiles with reference *B. sphaericus*

| **Numeric Names of Fatty Acids (Peak names)** | **Percent % MBI 5** | **Percent % *B.sphaericus*** |
|---|---|---|
| 14:0 iso | 2,02 | 1,26 |
| 14:0 | 0,60 | 0,85 |
| 15:0 iso | 45,00 | 46,61 |
| 15:0 anteiso | 10,87 | 7,89 |
| 14:0 iso 3OH | - | 1,05 |
| 16:1 w7c alcohol | 9,93 | 6,80 |
| 16:iso | 12,65 | 5,48 |
| 16:1 w11c | 3,31 | 5,62 |
| 16:0 | 1,72 | 1,64 |
| 17:1 iso w10c | 1,43 | 4,92 |
| Sum In Feature 4 | 1,65 | 2,58 |
| 17:0 iso | 6,11 | 10,86 |
| 17:0 anteiso | 4,70 | 4,45 |
| 18:1 w9c | - | - |

BIOLOG assay results revealed that each bacteria use different metabolites which can be seen from the changes of microplates. With Biolog GP plates, positive identifications were obtained after 48 h incubation. MBI 5 was performed 0.470 similarities with *B.sphaericus.* The results of FAME and BIOLOG analysis are not enough to have an accurate decision about bacterial species so that 16S-rRNA gene sequencing was performed for the identification of bacterial strains.

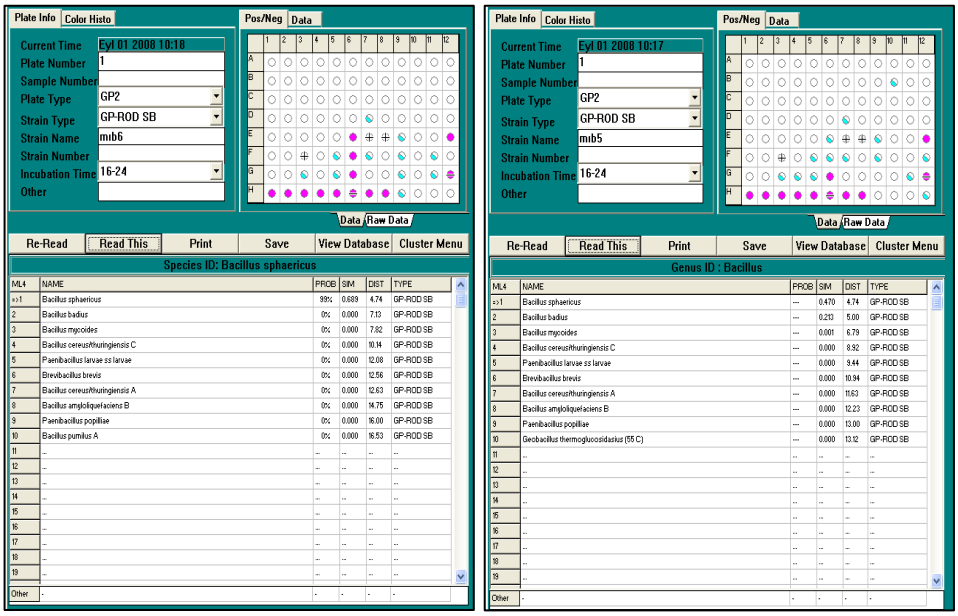


Figure 4.2. The results of BIOLOG micro assay of MBI5 and reference *B. sphaericus*

According the sequencing results and phylogenetic tree, MBI5 differ from known *Bacillus* species. All these results show that MBI5 is a new strain that have remarkable mosquitocidal effects against larvae.

Moreover, molecular identification of MBI5 strain was performed. Firstly, genomic DNA isolated from MBI5 (Figure 4.4). All genomic DNA of strains isolated well.

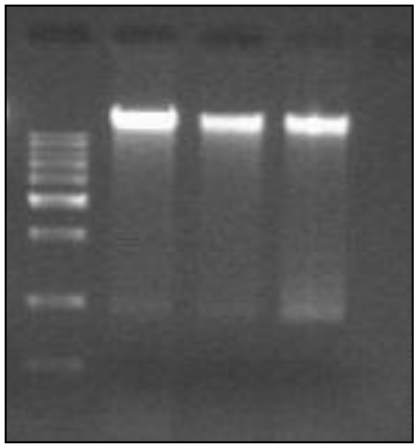

Figure 4.3. Agarose gel result of genomic DNA isolation from MBI5 and the marker (10 kb)

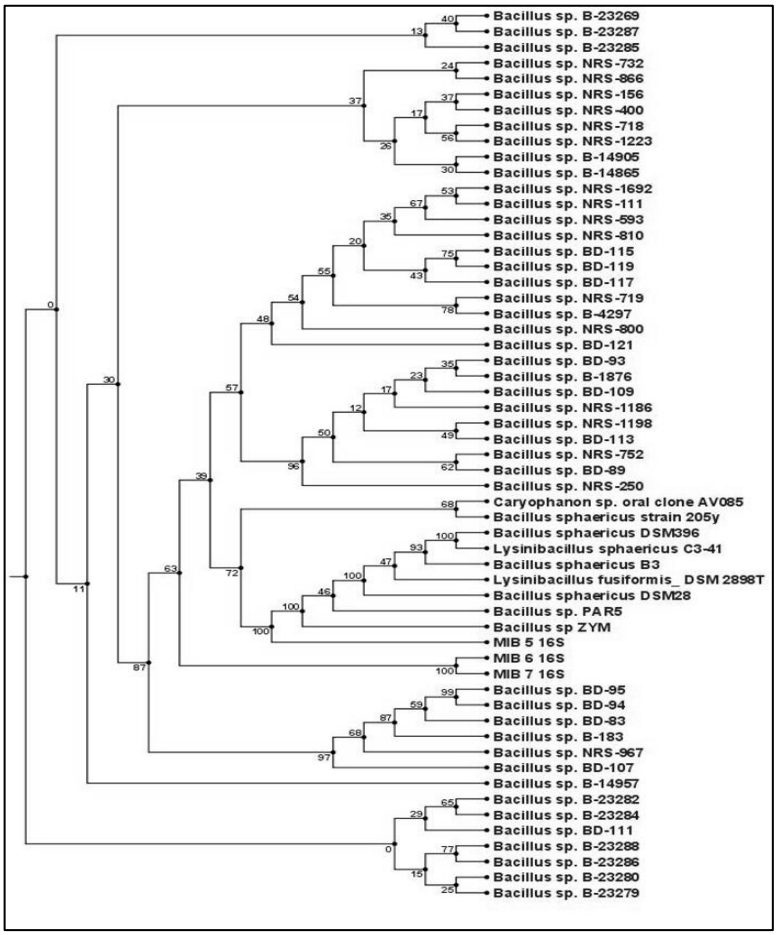


Figure 4.4. Phylogenetic tree of MBI5 according to 16S-rRNA gene sequencing

The genes were cloned from the bacterium (MBI5) and considered the genome of MBI5 to perform further experiments such as molecular cloning and protein isolation. After isolation of genomic DNA from MBI5, gradient PCR was performed using new designed primers for both BinA and BinB genes to find out best Tm values (Figure 4.5). According to results, the 55 °C was found to be the optimum temperature degree for both genes.

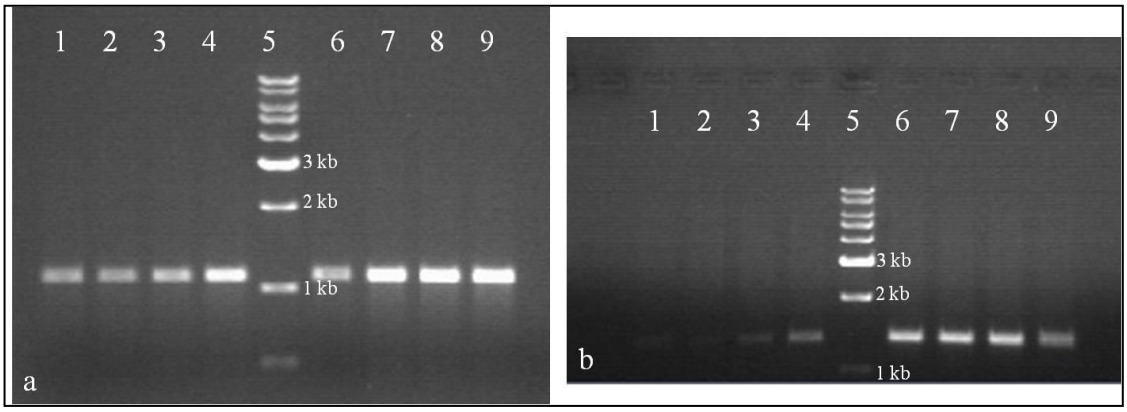


Figure 4.5. Agarose gel result of gradient PCR results. (a) BinA gene, (b) BinB gene. Line 1, 2, 3, 4, 6, 7, 8, 9 indicate the annealing temperature 48, 48.5, 49.5, 50.9, 52.8, 54.4, 55.4, 56 °C, respectively. Line 5 shows the marker (10 kb)

## 4.2. CLONING OF BINA AND BINB GENES

The BinA (1112 bp) and BinB (1346 bp) genes from MBI5 were cloned into pET-16b (5711 bp) plasmid individually to overexpress the toxin proteins. In order to provide sticky ends in PCR products of the genes, the primers were designed by adding restriction side (see section 3.9) which consisting by NdeI and BamHI. These restriction sides are also found in pET-16b plasmid.

Firstly, the BinA and BinB genes amplified by PCR (Figure 4.5). The PCR product was blunt end at this stage and it was not possible to clone target genes into plasmid. Therefore, the PCR product was cleaned and the genes were digested with NdeI and BamHI restriction enzymes to make sticky end of genes. The plasmid, simultaneously, was cut by NdeI and BamHI restriction enzymes to prepare the plasmid for cloning (Figure 4.6).

The digested plasmid and PCR product were incubated under appropriate conditions. The obtained plasmid-gene construct was transformed to *E. coli* DH5α and was grown in LB medium containing ampicillin antibiotic. In order to check whether cloning was successful or not, the colonies on plate was performed by colony PCR method and several colonies obtained from both genes (Figure 4.7).

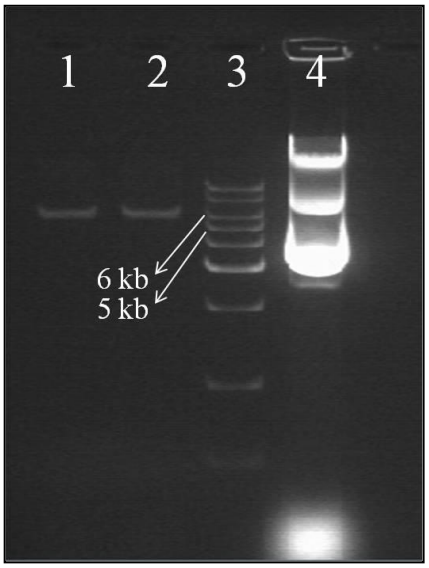


Figure 4.6. Agarose gel result of digested plasmid by (1) BamHI and (2) NdeI, (4) Uncut plasmid and (3) the marker (10 kb) were also showed

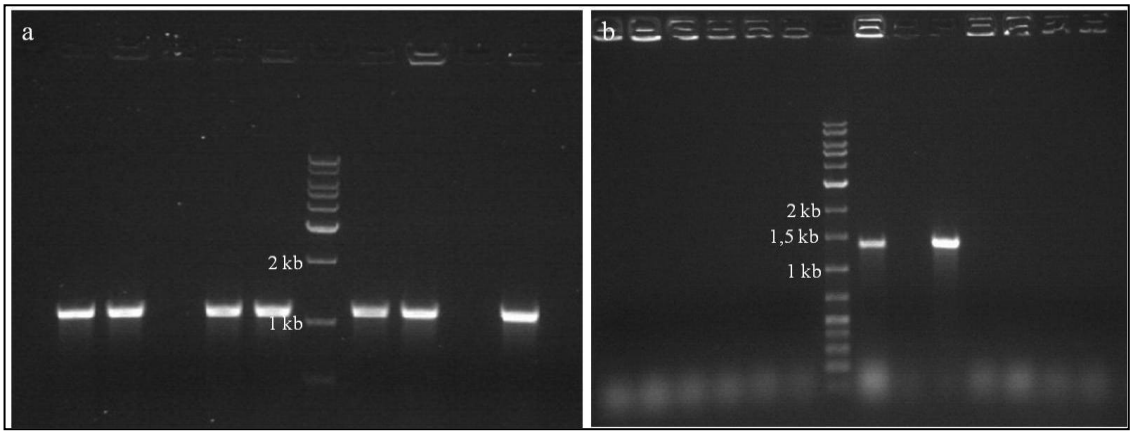


Figure 4.7. Colony PCR results of (a) BinA and (b) BinB gene

As a second confirmation to verify plasmid-gene construct, the construct plasmids were isolated from the colonies and cut with NdeI and BamHI restriction enzymes (Figure 4.8), therefore, it would be a strong evidence to start protein expression. The results showed that both BinA and BinB were cloned into pET-16b plasmid successfully. In this stage, invert inserting possibility did not study because our PCR products were prepared with sticky end as well as plasmid. One another factor is that the cloning was performed by double digesting so that it was not possible to bind of the free sides of plasmid each other.

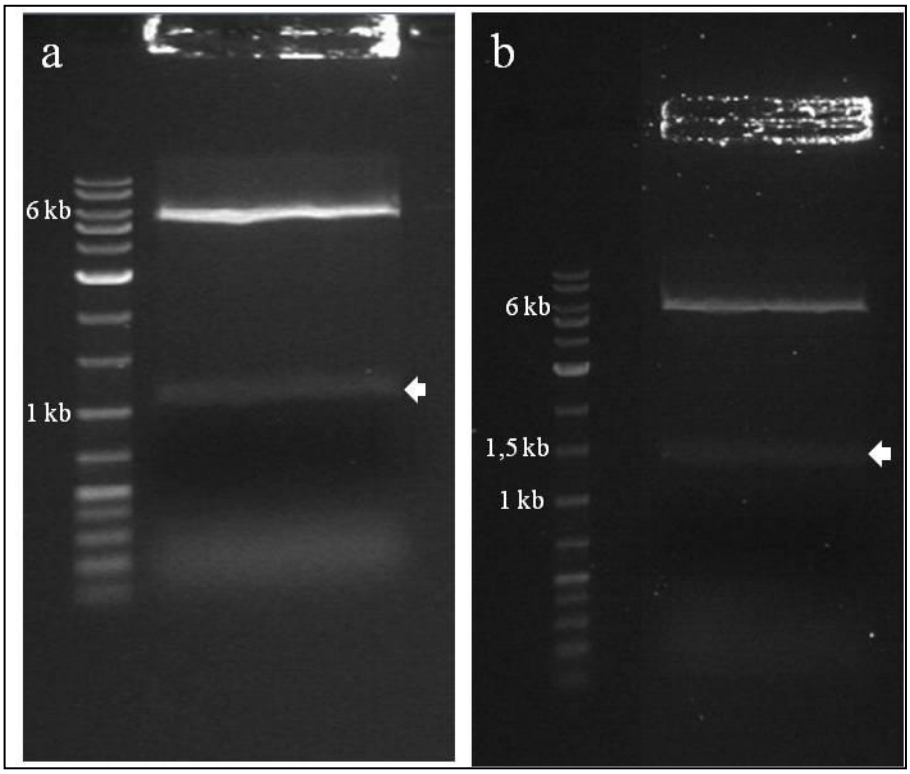


Figure 4.8. Construct plasmid digestion. (a) pET-16b+BinA, (b) pET-16b+BinB. Arrows indicates the inserts of plasmid.

After all verification, the obtained plasmids from *E coli* DH5α transformed into *E. coli* BL21 and colony PCR was performed again before starting protein expression. The plasmid didn't isolate from *E. coli* BL21 because of existing high endonuclease activity of *E. coli* BL21 during isolation. All steps of cloning were passed after getting the results of verification and verified plasmids was stored at -20 °C.

## 4.3. PURIFICATION OF THE BINA AND BINB TOXIN PROTEINS

As construct plasmids transformed into *E. coli* BL21, the toxin proteins were overexpressed separately. To find out best expression level of toxin proteins in total protein, several hours incubation periods were conducted after adding of IPTG to the culture. In this experiment, 0,4 mM IPTG was used and 1 h, 2h, 3h, 4 h, 5 h and overnight incubation periods were performed to find out the best expression time. It was detected that 4 h expression time was best for the pET-16b + BinA (BinB) construct. (Figure 4.9 and Figure 4.10)

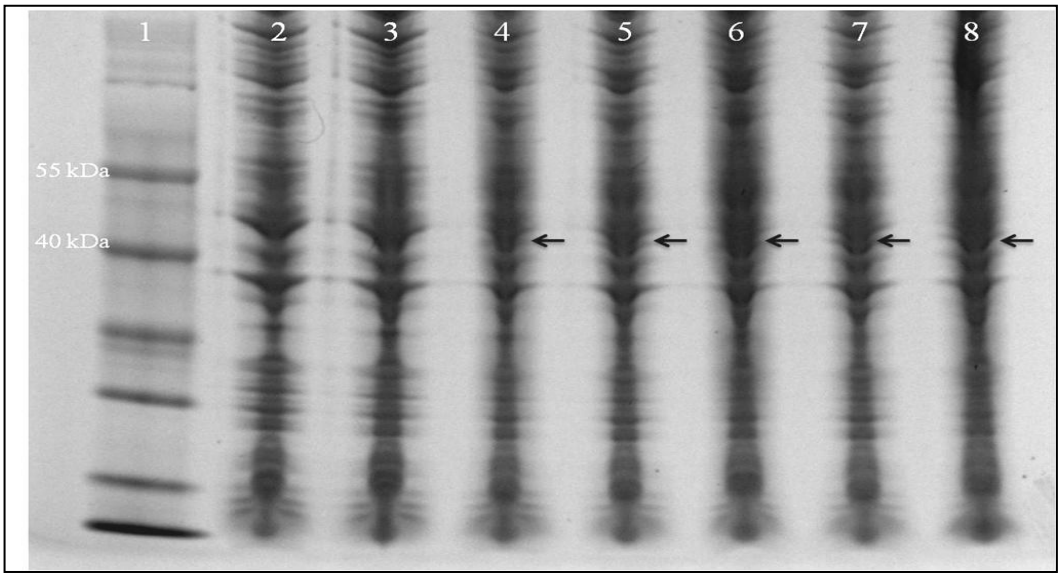


Figure 4.9. 12% SDS-PAGE analysis of BinA overexpression in *E. coli* BL21, (1) uninduced cell and (3, 4, 5, 6, 7, 8) indicates the expression time of 1h, 2h, 3h, 4h, 5h and overnight, respectively as well as (1) the marker. The BinA protein is marked with an arrow

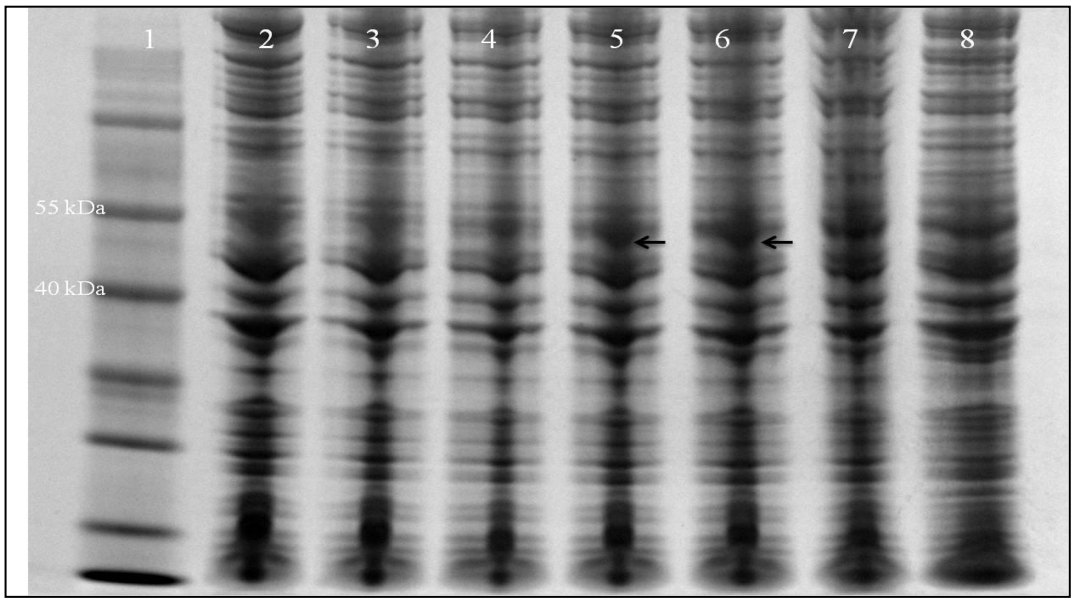


Figure 4.10. 12% SDS-PAGE analysis of BinB overexpression in *E. coli* BL21, (1) uninduced cell and (3, 4, 5, 6, 7, 8) indicates the expression time of 1h, 2h, 3h, 4h, 5h and overnight, respectively as well as (1) the marker. The BinB protein is marked with an arrow

After finding that 4 h inoculation of bacteria culture with 0,4 mM IPTG, a new experiment was set to obtain a big scale of total protein. The total protein includes both BinA and BinB was then purified using resin that has affinity against His-tag which both of toxin proteins were also carried His-tag. During SDS-PAGE, total protein from uninduced bacteria was used to understand the protein expression of target proteins. When the incubation time finished, induced bacteria was also used at SDS-PAGE. Besides, a sample before performing resin and after resin was used as well as purified protein. (Figure 4.11). Both protein were purified using affinity consist both proteins and resin.

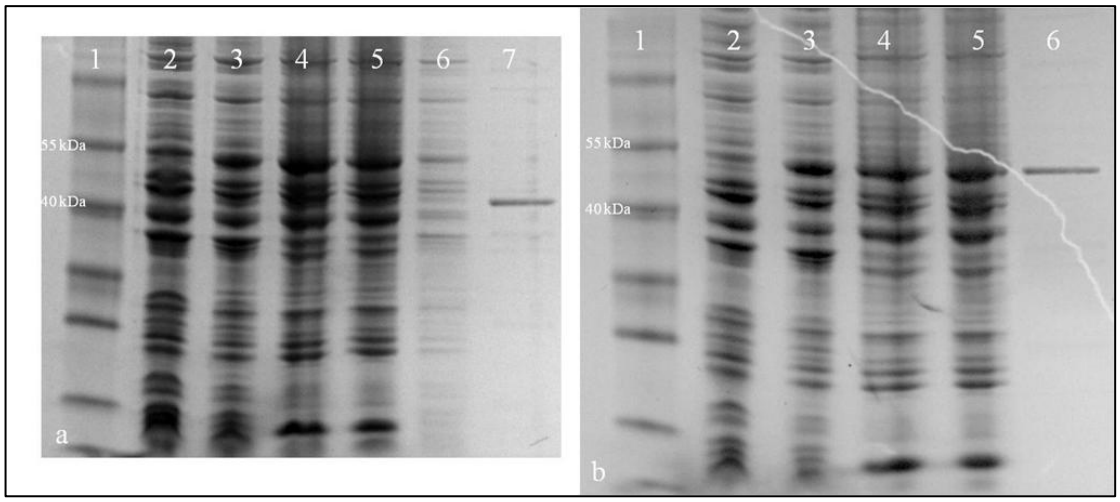


Figure 4.11. 12% SDS-PAGE analysis of both purified (a) BinA and (b) BinB. (a) (2,3) uninduced cell, induced cells, respectively and (4,5) a sample before and after resin, (6) experimental error and (7) indicates purified BinA protein. (b) (2,3) uninduced cell, induced cells, respectively and (4,5) a sample after and before resin. (6) indicates purified BinB protein. Both (1) shows the marker

## 4.4. BIOASSAY TEST OF MBI5

According to the bioassay test results MBI5 has a potential to be toxic to larvae of *Culex spp*. Investigation of larvacidal features of MBI bacterium was done in fresh and polluted water that contained 100 larvae (Table 4.2). Bti ATCC 35646, Bti 4Q4 and commercial *B.sphaericus* were used as positive control. The Bti strains were less effective on 2$^{nd}$ or 3$^{rd}$ instar larvae with respect of strains of *B.sphaericus*.

Table 4.2. Bioassay test results of MBI 5, *B.sphaericus*, Bti ATCC 35646 and Bti 4Q4 against live larvae number of *Culex spp*. at polluted/fresh water

| **Bacteria** | **Alive number larvae of Culex spp. (Total number : 100 in 500ml)** | | |
|---|---|---|---|
| | **Polluted water** | | **Fresh water** |
| | **24 h** | **48 h** | **24 h** |
| MBI5 | 6 | 4 | 0 |
| *B. sphaericus* | 10 | 4 | 0 |
| Bti ATCC 35646 | 20 | 20 | 16 |
| Bti 4Q4 | 34 | 32 | 24 |

## 4.5. TOXICITY OF BINARY PROTEINS

Purified binary proteins were applied to *Culex pipiens* larvae to test toxicity effect. Larvae were treated with the BinA, BinB and a combination of both proteins. In Table 4.2, BinA has toxic affect against larvae. There is an increase of total dead larvae from 7. to 12. hours with BinA protein treatment.

Table 4.3. The total dead larvae of MBI5, BinA, BinB and combinations of proteins for hours

| | **Hours** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **6** | **7** | **8** | **9** | **10** | **12** | **24** | **48** |
| BinA | 0 | 4 | 15 | 26 | 28 | 30 | 30 | 30 |
| BinB | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| BinA+BinB | 0 | 5 | 18 | 27 | 29 | 30 | 30 | 30 |

However, it can be inferred from the table that alone BinB is not toxic against larvae. In contrast, the combination of BinA and BinB proteins show that there is an increase the

total dead larvae when compared with alone BinA treatment. All the components except BinB showed toxicity until 12. h of experiment.

The toxicity levels of proteins were calculated by $LC_{50}$ method according to 8. hour. Both BinA and BinB were applied with 185, 200 and 215 ng/ml concentration and the $LC_{50}$ value was calculated 206,8 ng/ml. that means, BinA is highly toxic against *Culex*. In combination of protein group, three different concentrations (385, 400 and 415 ng/ml) were apllied. In this stage, 1:1 molar ratio concentration was used from each protein. When comparing of $LC_{50}$ value of combination proteins (Table 4.3) and the total dead larvae amount (Table 4.2), revealed that the combination of proteins are not highly toxic compared to alone BinA protein.

Table 4.4. $LC_{50}$ values of purified BinA and BinB protein against 2-3 instar larvae of *Culex pipiens*.

| | **LC (ng/ml)** | | |
|---|---|---|---|
| **Protein** | **%50** | **Lower** | **Upper** |
| | | | |
| BinA | 206.816 | 195.977 | 231.754 |
| BinB | Not toxic | - | - |
| BinA+BinB | 379.731 | 352.951 | 388.896 |

This study showed that a novel bacterium (MBI5) and its toxin proteins have remarkable toxicity against *Culex*.

# 5. DISCUSSION

Bacterial insecticides have been tested with limited use for the control of vector mosquitoes for more than five decades [74]. Because, it is clear that there is an urgent need for new agents and strategies to control mosquito-borne diseases. Potential strategies include vaccines and transgenic mosquitoes refractive to the causative disease agents, but, in the near future, control efforts will rely on insecticides. Significantly, the prospects for developing recombinant bacteria with high efficacy suitable for commercial development have improved recently due to the availability of genetic elements for improving endotoxin synthesis, a greater range of mosquitocidal proteins and the development of a better understanding of the toxicological properties of Bin protein [75].

Early studies reported that the toxicity of binary proteins of *B. sphaericus* are high against larvae of *Culex* and *Anopheles* mosquitoes, but low or nontoxic to *Aedes* larvae [30, 76]. In contrast, *B. thrungiensis* is more active against *Aedes* and *Culex* . and less active against *Anopheles*. Besides, *B. sphaericus* are able to live under polluted aquatics environments but *B.thrungiensis* is not able to work under polluted environments due to organic components. From this view, recombinant DNA techniques have been used to improve bacterial insecticide efficacy by increasing the synthesis of mosquitocidal proteins and enabling toxin combinations from different bacteria produced within single strains [49, 77].

In this study, a new mosquicidal strain (MBI5) was identified and characterized. The FAME analysis showed that MBI5 strain differ from commercially *B. sphaericus* by having the major cellular fatty acids which iso-pentadecanoic acid ($C_{15:0}$ iso, 45,00%) and $C_{16:0}$ iso, 12,65%. Moreover, minor amounts of the iso-branched fatty acids $C_{14:0}$ iso (0.60%), $C_{16:0}$ (1.72%), $C_{17:1}$ iso ω10c (1,43%). BIOLOG assay results also revealed that each bacteria use different metabolites. The phylogenetic tree showed that MBI5 strain differ from known *Bacillus* species (see section 4.1). According to the FAME, BIOLOG and 16S-rRNA analysis, it is concluded that MBI5 is a new mosqucidal strains. To analyze the toxin proteins of strains, primers were designed and it was observed that the strains have both BinA and BinB genes (see section 4.1).

MBI5 strain was used to test to toxicity with toxin proteins but the $LC_{50}$ value did not calculated of this bacterium. The experiment was set up in 250 ml flask with $10^8$ cfu/ml bacteria injection into flask containing 100 larvae totally in both fresh and polluted water. It is observed that MBI5 strain killed 94 larvae among 100 in polluted water during 24 hour period after injecting of bacteria. Commercial *B. sphaericus*, Bti ATCC 35646 and Bti 4Q4 showed less toxicity against *Culex spp*. It is concluded that the newly isolated *B. sphaericus* has strong insectical activity than known reference strains.

Binary toxins (BinA and BinB) cloned from newly isolated *B. sphaericus* (MBI5) into plasmid (pET-16b) and expressed into *E. coli* to test the toxicity effect of toxins. According to $LC_{50}$ values of alone BinA, the $LC_{50}$ value was calculated 206,8 (195,9-231,7) ng $ml^{-1}$ for 8. hour. The concentration of BinA applied in high concentration and the result showed that purified soluble alone BinA protein is toxic against *Culex pipiens*. Our results show similarity with a recent study [78] which used purified BinA protein with and without poly-histidine tag showed $LC_{50}$ dose of 82,3 and 66,9 ng $ml^{-1}$, respectively, at 48 h against *Culex*. One another important factor is that working soluble BinA protein shows that the proteins folded properly which was not investigated in this study.

In our experiment, alone BinB is not toxic against *Culex* which was expected as reported in previous many studies [52, 53].

Larvacidal activity of BinA and BinB combination at 1:1 molar ratio was also studied. The $LC_{50}$ values of BinA-BinB mixture was calculated 379,7 (352,9-388,8) ng $ml^{-1}$. In Table 4.2, it can be concluded that the BinA-BinB mixture were caused more rapid mortality of *Culex* larvae than BinA. Nevertheless, the mortality is not enough to report that the BinA-BinB mixture has a high toxicity effect on larvae because of using high amount of protein at BinA-BinB mixture. In literature, it is reported [79] that $LC_{50}$ of BinA-BinB mixture was calculated as 8.0 (5,7 – 10,4) ng $ml^{-1}$ at 48 hours against *C. quinquefasciatus,* which has quite higher toxicity than our protein mixture. These controversy results may be explained by the low efficiency of BinB protein in larvae midgut.

It is concluded that the MBI5 strain and its binary proteins have insectical activity against *Culex spp.* larvae. Side effects of MBI5 strain were carried out on rats with toxicology

experiments in previous. It was not observed that change of feeding levels**,** water consumption, differences in physiological behavior and, rising of heaviness depending on time were not observed on laboratory rats. Moreover, it was reported that the blood, liver enzyme and microscopic pathological symptom such as liver, craw, splenetic, kidney and small intestine analysis of laboratory rats were normal.

# 6. CONCLUSION

Using entomopathogens as biopesticides can reduce the use of synthetic pesticides. Entomopathogens have been isolated from soils and the carcasses of insects. Bacillus species is currently used as a biopesticide with mosquitocidal action involving protein toxins, notably, binary toxins.

In conclusion, we have showed that binary proteins of a novel *B. sphaericus* (MBI5) could be expressed in *E. coli* heterologously. The proteins are worked well for toxicology assay against mosquito larvae. The purified BinA alone showed high toxicity towards larvae of *Culex sp.*, but not BinB.

Since the larvae were fed with the BinA protein, higher toxicity dose used and reported in this study. The protocols were prepared of purification of binary toxins of *B. sphaericus* as active form for further studies in this area.

This study demonstrates a new isolated *B. sphaericus* (MBI5) strain can be used as a mosquitocidal agent against *Culex spp*. Using biological agents instead of synthetic pesticides will lead to decrease the usage of chemicals on Earth.

# 7. FUTURE DIRECTIONS

As a future work about this project, the both binary genes isolated from *B. sphaericus* (MBI5) can be cloned into a plasmid which has a strong promoter and, therefore, this plasmid can introduce into another mosquicidal bacteria *B. thuringiensis*, which has active cry genes. It is expected to increase the toxicity level of bacteria and kill the broad range of mosquito using just one bacteria strain.

Another work is that different concentration of binary toxins that isolated from MBI5 must be used against mosquitoe larvae so that it can be founded the minimal protein dose to test letal effect on larvae.

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