



Final Report

**Effective compounds from *Xenorhabdus* and
Photorhabdus isolates from Thailand against mosquito
larvae**

By Dr. Aunchalee Thanwisai

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**Effective compounds from *Xenorhabdus* and *Photorhabdus*
isolates from Thailand against mosquito larvae**

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Abstract

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Project Title : Effective compounds from *Xenorhabdus* and *Photorhabdus* isolates from Thailand against mosquito larvae

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Abstract:

เพื่อศึกษาประสิทธิภาพของ Whole cell suspension, Cell-free supernatant, Crude cell extract และ Bacteria cell pellet จากแบคทีเรีย *Xenorhabdus* และ *Photorhabdus* ที่พบในประเทศไทยต่อการฆ่าลูกน้ำยุงลาย (*Aedes*) ยุงรำคาญ (*Culex*) และยุงก้นปล่อง (*Anopheles*) ในระยะ 1-2 และระยะ 3-4

ทำการทดสอบ Whole cell suspension, Cell-free supernatant, Crude cell extract และ Bacteria cell pellet จากแบคทีเรีย *Xenorhabdus* และ *Photorhabdus* กับลูกน้ำยุงลาย (*Aedes*) ยุงรำคาญ (*Culex*) และยุงก้นปล่อง (*Anopheles*) ในระยะ 1-2 และระยะ 3-4 กลุ่มควบคุมคือ *Escherichia coli*, อาหารเพาะเชื้อ LB, Phosphate buffer saline (PBS) และ Distilled water แต่ละการทดลองทำ 2 ซ้ำ ในแต่ละการทดลองใช้ลูกน้ำยุง 10 ตัวในน้ำ 20 มิลลิลิตร ทำการหยด Whole cell suspension ปริมาตร 200 มิลลิลิตร, Cell-free supernatant ปริมาตร 200 มิลลิลิตร, Crude cell extract ปริมาตร 500 มิลลิลิตรและ Cell pellet ปริมาตร 500 มิลลิลิตร ลงในน้ำที่มีลูกน้ำยุง สังเกตอัตราการตายของลูกน้ำยุงที่เวลา 24,48,72,96 และ 120 ชั่วโมง

ประสิทธิภาพของ Whole cell suspension จากแบคทีเรีย *Photorhabdus luminescens* subsp. *hainaensis* (KK1.3) และ *Xenorhabdus miraniensis* (PB62.2) มีประสิทธิภาพในการ

ฆ่าลูกน้ำยุงลาย (*Aedes*) และยุงรำคาญ (*Culex*) ในระยะ 1-2 ได้ดีที่สุด ในขณะที่ Whole cell suspension จากแบคทีเรีย *Photorhabdus luminescens* subsp. *hainaensis* (KK1.3) *Photorhabdus luminescens* subsp. *akhurstii* (NK2.5) ก็มีประสิทธิภาพในการฆ่าลูกน้ำยุงรำคาญ (*Culex*) ในระยะ-3-4 ได้ดีเช่นเดียวกัน สำหรับ Cell free supernatant จากแบคทีเรีย *Photorhabdus luminescens* subsp. *hainaensis* (KK1.3) มีประสิทธิภาพในการฆ่าลูกน้ำยุงลาย (*Aedes*) ในระยะ 1-2 ได้ดีที่สุด ในขณะที่ Cell free supernatant จากแบคทีเรีย *Xenorhabdus miraniensis* (PB62.2) และ *Xenorhabdus stockiae* (PB57.4) มีประสิทธิภาพในการฆ่าลูกน้ำยุงรำคาญ (*Culex*) ในระยะ-3-4 ได้ดี สำหรับ Crude cell extract จากแบคทีเรีย *Xenorhabdus stockiae* (PB57.4) และ *Photorhabdus luminescens* subsp. *akhurstii* (NK2.5) มีประสิทธิภาพสามารถฆ่าลูกน้ำยุงรำคาญ (*Culex*) ในระยะ-3-4 ได้ดี และ Bacteria cell pellet จากแบคทีเรีย *Photorhabdus luminescens* subsp. *hainaensis* (KK1.3) มีประสิทธิภาพสามารถฆ่าลูกน้ำยุงลาย (*Aedes*) และลูกน้ำยุงรำคาญ (*Culex*) ในระยะ 1-2 ได้ดี ในขณะที่ Bacteria cell pellet จากแบคทีเรีย *Photorhabdus luminescens* subsp. *hainaensis* (KK1.3) *Photorhabdus luminescens* subsp. *akhurstii* (NK2.5) มีประสิทธิภาพสามารถฆ่าลูกน้ำยุงรำคาญ (*Culex*) ในระยะ3-4 ได้ดีเช่นเดียวกัน ส่วน Bacteria cell pellet จากแบคทีเรีย *Xenorhabdus stockiae* (PB10.5 และ PB57.4) มีประสิทธิภาพในการฆ่าลูกน้ำยุงก้นปล่อง (*Anopheles*) ในระยะ 2 ได้

จากการศึกษาที่ผ่านมา *Xenorhabdus nematop* สามารถผลิตสาร หรือสารพิษ หรือ ipopolysaccharides ที่มีคุณสมบัติฆ่าแมลงได้ ซึ่งเป็นไปได้ว่าแบคทีเรีย *Xenorhabdus* และ *Photorhabdus* ที่แยกได้ในประเทศไทย สามารถผลิตสารต่างๆ เหล่านี้ได้ อย่างไรก็ตาม สารประกอบบริสุทธิ์ที่ผลิตโดยแบคทีเรียทั้งสองจึงจำเป็นต้องศึกษาในขั้นต่อไป

An alternative strategy to control insect pests and vectors are required for agriculture and control of infectious diseases. Most of the insecticidal toxins which have been used in agriculture are derived from Gram-negative bacteria, *Bacillus thuringiensis* or *Bt*, however, a number of studies reported the development of insect resistance against *B. thuringiensis* toxin.

Insecticide activity of whole cell suspension, cell-free supernatant, crude cell extract and bacteria cell pellet of *Xenorhabdus* and *Photorhabdus* against larva stage 1-2 and 3-4 of *Aedes*, *Culex* and *Anopheles*

Whole cell suspension of *Photorhabdus luminescens* subsp. *hainaensis* (KK1.3) and *Xenorhabdus miraniensis* (PB62.2) showed high effective to kill larva stage 1-2 of *Aedes* and *Culex*. Furthermore, Whole cell suspension of *Photorhabdus luminescens* subsp. *hainaensis* (KK1.3) and *Photorhabdus luminescens* subsp. *akhurstii* (NK2.5) showed effective to kill larva stage 3-4 of and *Culex*. Cell free supernatant of *Photorhabdus luminescens* subsp. *hainaensis* (KK1.3) showed high effective to kill larva stage 1-2 of *Aedes*. In addition, cell free supernatant of *Xenorhabdus miraniensis* (PB62.2) and *Xenorhabdus stockiae* (PB57.4) showed high effective to kill larva stage 1-2 of *Aedes* and larva stage 3-4 of *Culex*. Crude cell extract of *Xenorhabdus stockiae* (PB57.4) and *Photorhabdus luminescens* subsp. *akhurstii* (NK2.5) can kill larva stage 3-4 of *Culex*, whereas, bacteria cell pellet of *Photorhabdus luminescens* subsp. *hainaensis* (KK1.3) and *Photorhabdus luminescens* subsp. *akhurstii* (NK2.5) can also kill larva stage 3-4 of *Culex*. Furthermore, bacteria cell pellet of 2 isolates of *Xenorhabdus stockiae* (PB10.5 and PB57.4) can kill larva stage 2 of *Anopheles*.

Previously study, *Xenorhabdus nematophila* can produce compounds with insecticidal properties include toxin complexes (Tc's) or lipopolysaccharides. These isolates may be due to can produce bioactive compounds that are effective in killing mosquito larva and an alternative biological agents for *Aedes*, *Culex* and *Anopheles*. However, the isolation of compounds produced by *Xenorhabdus* and *Photorhabdus* are needed to investigate for more understanding.

Keywords : *Xenorhabdus*, *Photorhabdus*, insecticidal activity; mosquitoes larva

Executive summary

Biological control is widely used for controlling agricultural pests for several years since the hazardous of chemicals. These chemicals remain in the food chain. Currently, people use toxins from of *Bacillus thuringiensis* (*Bt*) for the control of mosquito larvae, however, several studies reported the development of insect resistant against *B. thuringiensis* toxin (McGaughey et al., 1985; McGaughey et al., 1992; Chattopadhyay et al., 2004). Therefore, alternative strategies to control mosquito larvae are needed.

To investigate the killing effect of whole cell suspension, cell-free supernatant, crude extract, bacterial cell pellet and recombinant pirA/pirB protein from *Xenorhabdus* and *Photorhabdus* bacteria from Thailand on *Aedes*, *Anopheles* and *Culex* mosquitoes larvae.

Whole cell suspension of *Photorhabdus luminescens* subsp. *hainaensis* (KK1.3) and *Xenorhabdus miraniensis* (PB62.2) showed high effective to kill larva stage 1-2 of *Aedes* and *Culex*. Furthermore, Whole cell suspension of *Photorhabdus luminescens* subsp. *hainaensis* (KK1.3) and *Photorhabdus luminescens* subsp. *akhurstii* (NK2.5) showed effective to kill larva stage 3-4 of and *Culex*. Cell free supernatant of *Photorhabdus luminescens* subsp. *hainaensis* (KK1.3) showed high effective to kill larva stage 1-2 of *Aedes*. In addition, cell free supernatant of *Xenorhabdus miraniensis* (PB62.2) and *Xenorhabdus stockiae* (PB57.4) showed high effective to kill larva stage 1-2 of *Aedes* and larva stage 3-4 of *Culex*. Crude cell extract of *Xenorhabdus stockiae* (PB57.4) and *Photorhabdus luminescens* subsp. *akhurstii* (NK2.5) can kill larva stage 3-4 of *Culex*, whereas, bacteria cell pellet of *Photorhabdus luminescens* subsp. *hainaensis* (KK1.3) and *Photorhabdus luminescens* subsp. *akhurstii* (NK2.5) can also kill larva stage 3-4 of *Culex*. Furthermore, bacteria cell pellet of 2 isolates of *Xenorhabdus stockiae* (PB10.5 and PB57.4) can kill larva stage 2 of *Anopheles*. However, protein (toxin) from recombinant PirA and PirB gene cannot express. It may be toxic to bacterial cell.

From this result, these isolates may be due to can produce bioactive compounds that are effective in killing mosquito larva. Previously study, *Xenorhabdus nematophila* can produce compounds with insecticidal properties include toxin complexes (Tc's) or lipopolysaccharides (Owuama, 2001; Ffrench-constant, et al., 2007; Hinchliffe, et al., 2010). This indicates that *Xenorhabdus* and *Photorhabdus* may be an alternative biological agents for *Aedes*, *Culex* and *Anopheles*. However, the isolation of compounds produced by *Xenorhabdus* and *Photorhabdus* are needed to investigate for more understanding.

Introduction

Biological control is widely used for controlling agricultural pests for several years since the hazardous of chemicals. These chemicals remain in the food chain. Currently, people use toxins from of *Bacillus thuringiensis* (*Bt*) for the control of mosquito larvae, however, several studies reported the development of insect resistant against *B. thuringiensis* toxin (McGaughey et al., 1985; McGaughey et al., 1992; Chattopadhyay et al., 2004). Therefore, alternative strategies to control mosquito larvae are needed.

Genome analysis of a Gram-negative bacterium which is a symbiont of EPNs, *P. luminescens* subsp. *laumondii* strain TT01 has revealed a several genes predicting toxins, hemolysins, lipase and proteases. These factors are important for symbiosis between bacteria, EPNs and insect hosts (Duchaud et al., 2003).

We are interested in the metabolites from *Xenorhabdus* and *Photorhabdus* spp. These bacteria are bacterial symbionts of entomopathogenic nematodes (EPNs) belonging to the genus *Steinernema* and *Heterorhabditis*, respectively. The general features of the life cycles of them are similar. They live in the intestine of the infective juvenile stage of nematodes. The nematodes enter the digestive tract of the larval stage of a diverse range of insects and subsequently penetrate into the hemocoel of the host insect. Upon entrance into the hemocoel, the nematodes release the bacteria into the hemolymph. The bacteria are released and secrete toxins that rapidly kill the insect larva. Enzymes are also produced from the bacteria that lead to the decomposition of the carcass, providing both the bacteria and the nematodes with enough food for survival and reproduction. During the final stages of development, the nematodes and bacteria reassociate and the nematodes subsequently develop into a non-feeding infective juvenile stage. The infective juvenile carrying the bacteria in its intestinal tract, then emerges from the insect carcass in search of a new insect host. Bactericidal products of *Photorhabdus* and *Xenorhabdus* can kill insects as well as prevent infections of the carcass by other bacteria.

Four major groups of toxin have been classified from *P. luminescens* and characterized. They are (i) 'Toxin complexes' (Tcs) which is encoded by the PAI I (pathogenicity island I) and have been identified as high molecular weight insecticidal toxins comprised of multiple subunits (Tca, Tcb, Tcc, and Tcd) (Brown et al., 1998). (ii) *Tc* toxins which are insecticidal toxins against the Colorado potato beetle, *Leptinotarsa decemlineata* and the sweet potato whitefly *Bemisia tabaci* (Blackburn et al., 2005). (iii) 'Photorhabdus insect related' (Pir) toxins. They are encoded by the *PirAB* gene, located

at plu4093-4092 (pirA) and plu4437-4436 (pirB) loci. PirA and PirB proteins have similarities to the δ -endotoxins from *B. thuringiensis* and a developmentally regulated protein from the beetle, *Leptinotarsa decemlineata* (Waterfield et al., 2005). PirAB from *P. luminescens* when expressed in *Escherichia coli* had the ability to kill *Galleria mellonella* (Waterfield et al., 2005) and PirAB from *P. asymbiotica* when expressed in *E. coli* against both *Aedes aegypti* and *Aedes albopictus* larvae (Ahantarig et al., 2009). The 'makes caterpillars floppy' toxins 1 (Mcf1) and 2 (Mcf2) are encoded by PAI II (pathogenicity island II), these toxins when expressed in *E. coli*, allow the bacterium to survive inside the insect and promote its death (Rodou et al., 2010). (iv) 'Photorhabdus virulence cassettes' (PVC), the protein products of PVCs can destroy insect hemocytes (Rodou et al., 2010).

A previous report demonstrated PirA and PirB toxins from *Photorhabdus asymbiotica* from undescribed origin had killing activity against dengue-vectors (*Aedes aegypti* and *Aedes albopictus*). Another study showed that the whole cell suspension, cell-free supernatant, crude cell extract from *X. stockiae* could be used as a biological control for *Luciaphorus* sp., a mushroom mite (Bussaman et al., 2012). Although, toxins from *Xenorhabdus* are numerous (Sheets et al., 2011), they have never been investigated for the control of the mosquito larvae.

Many species of mosquitoes cause vector-borne disease. *Anopheles* transmits human malaria. *Aedes aegypti* can spread the dengue fever, Chikungunya and yellow fever viruses. *Culex* is vectors of West Nile virus, filariasis, and Japanese encephalitis.

We hypothesized that *Photorhabdus* and *Xenorhabdus* isolates from Thailand may be a good resource for various insecticide compounds. In a previous study, we isolated and characterized 69 *Xenorhabdus* and 57 *Photorhabdus* spp. from across Thailand together with their associated nematode symbionts, and characterized their phylogenetic diversity. This study revealed the genetic diversity of *Xenorhabdus* and *Photorhabdus* spp. and described new subspecies and associations between EPNs and their bacterial symbionts.

We will investigate the killing effect of whole cell suspension, cell-free supernatant, crude cell extracts and pirA and pirB proteins from *Xenorhabdus* and *Photorhabdus* bacteria from Thailand. All extracts will be tested against three local mosquito larvae which are important transmission vectors for important diseases. The vectors include *Aedes*, *Anopheles* and *Culex* mosquitoes. The effective *Xenorhabdus* and *Photorhabdus* compounds will be identified further by chemical analysis.

Literature review

Xenorhabdus and *Photorhabdus* spp. are Gram-negative rods bacteria belonging to the family Enterobacteriaceae. They are motile, facultatively anaerobic, nonsporulating, and give oxidase-negative result. Both bacteria do not reduce nitrate and ferment only limited number of carbohydrates. Phylogenetic tree based on the 16S rDNA (Spröder et al., 1999), placed both genera in Enterobacteriaceae, related to *Proteus*. *Xenorhabdus* and *Photorhabdus* are similar in numerous characteristics. *Xenorhabdus* is specifically found associated *Steinernematidae*, while *Photorhabdus* is only associated with *Heterorhabditidae*. Both bacterial have never been isolated directly from soil (Akhurst and Boemare, 1990).

A primary characteristic distinguishing *Photorhabdus* from *Xenorhabdus* is the ability of the former to emit light under stationary phase culture conditions and in the infected host insect (Poinar et al., 1980). *Xenorhabdus* do not produce enzyme catalase, which is an usual characteristic of bacteria in the Enterobacteriaceae family (Boemare and Akhurst, 1988). Both bacteria can be cultured in laboratory conditions. During entry to stationary phase of their growth cycle, the bacteria secrete several extracellular products, including lipase, phospholipase, protease, and several different broad spectrum antibiotics into the insect hemolymph (Akhurst and Boemare, 1990). The degradative enzymes break down the macromolecules of the insect cadaver to provide the development of nematode with a nutrient supply, while the antibiotic products suppress contamination of the cadaver with other microorganisms. Cytoplasmic inclusion bodies, composed of highly expressed crystalline proteins, are also produced by both bacteria during stationary phase conditions (Couche et al., 1987). The pathogenic potential of the bacteria and nematode complex has been exploited for its usefulness as biological pest control agents (Klein, 1990).

Another intriguing property of *Xenorhabdus* and *Photorhabdus* is the formation of phenotypic variants forms. Phase I represents the form of the bacteria that is naturally associated with the infective juvenile nematode. The variant form, or phase II cell, alters many properties and is not found as natural symbionts in the nematode (Akhurst, 1980). Phase II exhibit different colony morphologies from phase I, however, it maintains pathogenicity to *G. mellonella* host. Other differences are that phase I produce antibiotics, absorbs certain dyes, and develops larger intracellular inclusion than phase II.

P. asymbiotica is the only species of *Photorhabdus* known to be a human pathogen. It is associated with invasive soft tissues and disseminates bacteraemic infections in human patients. A few reports from United state and Australia (Weissfeld et al., 2005; Gerrard et al., 2006) indicate that the organism can be isolated from soft tissue such as skin or blood samples of the patients whose infection were transmitted through *H. gerrardi* nematode (Plichta et al., 2009). This bacterium grows on conventional bacterial culture media (Gerrard et al., 2004).

8.2 Life cycle of *Xenorhabdus* and *Photorhabdus* bacteria

General features of life cycles of *Xenorhabdus* and *Photorhabdus* are similar in the way that they are symbiotic with nematodes and pathogenic for insects. There are two different physiological states in the life cycle of the symbionts corresponding to two different ecological niches. The first is mutualism in which bacteria reside in the resting stage of the nematode. *Xenorhabdus* and *Photorhabdus* spp. are symbionts in the intestine of the infective juvenile stage of EPNs. The second stage is a vegetative state. The bacteria multiply inside the insects after inoculation in hemolymph and kill insect. It is called pathogenic stage (Figure 1). During their life cycle, both *Xenorhabdus* and *Photorhabdus* have pathogenic and mutualistic interactions with their different invertebrate hosts, i.e. the insect and nematode. The life cycle starts and ends with colonization of bacteria in the gut of EPNs. Nematode reproduction is optimum when the natural symbiont dominates the microbial flora, suggesting that the bacteria can serve as a food source and/or provide essential nutrients that are required for efficient nematode proliferation. During the final stages of development, the nematode and bacteria reassociate and the nematode subsequently develops into its non-feeding infective juvenile stage. All of the *Xenorhabdus* and almost all of the *Photorhabdus* isolates have been obtained from nematodes harvested from soil samples. Free-living forms of the bacteria have not yet been isolated from soil or water sources.

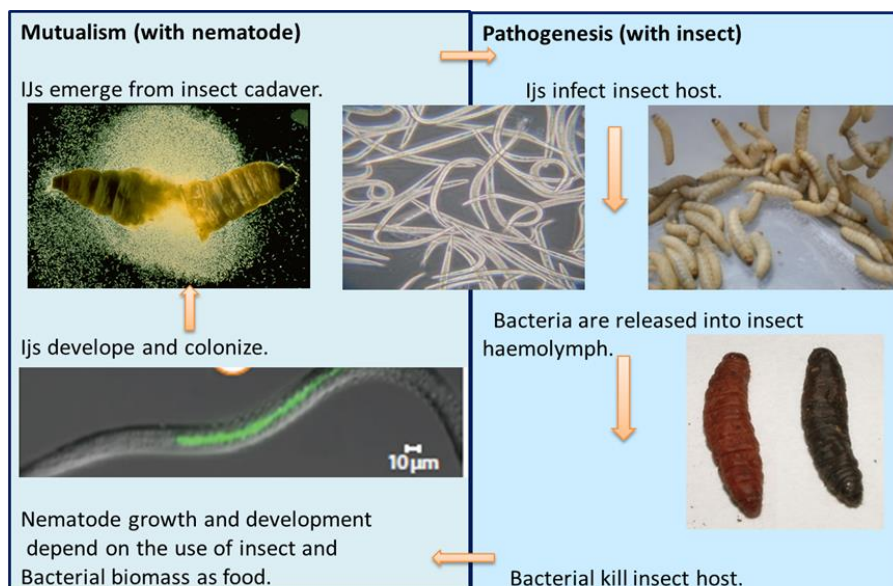


Figure 1 The life cycle of *Xenorhabdus* and *Photorhabdus* bacteria

8.3 Genomes of *Xenorhabdus* and *Photorhabdus* bacteria

Complete genome sequences of two of *Xenorhabdus* (*X. bovienii* SS-2004, and *X. nematophila* ATCC 1906), and two of *Photorhabdus* (*P. luminescens* subsp. *laumondii* strain TT01 and *P. asymbiotica*) are available (<http://www.ncbi.nlm.nih.gov/genome>) (Table 1).

X. bovienii SS-2004 possesses single chromosome of 4,225,498 bp with GC content of 44% and no plasmid. A total of 4,260 protein coding genes, include 113 of structural RNAs, and 42 of pseudogenes. *X. nematophila* ATCC 19061 genome is 4,432,590 bp with GC content of 44%. A total 4,299 protein coding genes include 109 structural RNAs and 206 pseudogenes. One plasmid, XNC1_p, with length 155,327 bp and GC content of 45% was present. It coded 175 proteins and 12 pseudogenes.

Genome of *Photorhabdus* is longer than that of *Xenorhabdus*. *P. luminescens* subsp. *laumondii* strain TT01 possesses a chromosome of 5,688,987 bp with GC content of 42% and no plasmid. A total 4,683 protein-coding genes include 107 structural RNAs and 222 pseudogenes. *P. asymbiotica* genome is shorter than that of *P. luminescens* subsp. *laumondii* strain TT0. The length of genome is 5,064,808 bp with GC content 42%. A total 4,390 protein-coding genes include 138 structural RNAs and 5 pseudogenes. A plasmid pPAU1 with 29,330 bp, GC content 40% was found. It contains 27 protein coding genes.

Table 1 Complete genomes and plasmids of *Xenorhabdus* and *Photorhabdus*

Strain	GenBank Accession number	Complete genome (Length)	Complete plasmid sequence (Length)	References
<i>X. bovienii</i> SS-2004	FN667741	4,225,498 bp	-	Chaston et al., 2011
<i>X. nematophila</i> ATCC 19061	FN667742	4,432,590 bp	-	Chaston et al., 2011
<i>X. nematophila</i> ATCC 19061 plasmid XNC1_p	FN667743	-	155,327 bp	Chaston et al., 2011
<i>P. luminescens</i> subsp. <i>laumondii</i> TTO1	BX470251	5,688,987 bp	-	Duchaud et al., 2003
<i>P. asymbiotica</i>	FM162591	5,064,808 bp	-	Wilkinson et al., 2009
<i>P. asymbiotica</i> plasmid pPAU1	FM162592	-	29,330 bp	Wilkinson et al., 2009

8.4 Identification of *Xenorhabdus* and *Photorhabdus* bacteria

Xenorhabdus and *Photorhabdus* spp. can be identified by conventional microbiological methods and biochemical tests. These bacteria are Gram-negative rods, motile, facultatively anaerobic, nonsporulating, and oxidase-negative. Molecular characterization of the 16S rDNA showed that *Xenorhabdus* was closely related to *Photorhabdus* and they are the sister group of the members of the family *Enterobacteriaceae* (Szallas et al., 1997).

Early researches used phenotypic data for taxonomy of *Xenorhabdus* and *Photorhabdus* bacteria. Five species were proposed by this method; *X. nematophila*, *X. beddingii*, *X. bovienii*, *X. poinarii* and *X. japonica*. However, the identification of new species is difficult because of high similarity in phenotypic characteristics, especially among members of *Xenorhabdus*. Some molecular methods such as PCR-RFLP, RAPD and ERIC based on 16S rDNA cannot discriminate in subspecies level completely. Sequence analysis of some housekeeping genes gave more reliable results for the taxonomy purpose. The phylogenetic analysis of *dnaN* or *recA* showed the promising topology for the discrimination of the members in two genera.

Twenty-one species of *Xenorhabdus* have been described (Table 2) (Tailliez et al., 2010; An and Grewal 2011). Genus *Photorhabdus* has 3 species, *P. luminescens*, *P. temperata* (Tailliez et al., 2010 and An and Grewal 2011) and *P. asymbiotica* (Fischer-Le Saux et al., 1999; Akhurst et al., 2004) (Table 2).

Table 2 Taxonomy of *Xenorhabdus* spp. and *Photorhabdus* spp.

Genus	Species and subspecies	References
<i>Xenorhabdus</i>	<i>nematophila</i>	Thomas and Poinar, 1979
	<i>beddingii</i>	Akhurst and Boemare 1988
	<i>bovienii</i>	Akhurst and Boemare 1988
	<i>poinarii</i>	Akhurst and Boemare 1988
	<i>japonica</i>	Nishimura et al., 1994
	<i>indica</i>	Nishimura et al., 1994
	<i>budapestensis</i>	Lengyel et al., 2005
	<i>ehlersii</i>	Lengyel et al., 2005
	<i>innexi</i>	Lengyel et al., 2005
	<i>szentirmaii</i>	Lengyel et al., 2005
	<i>budapestensis</i>	Lengyel et al., 2005
	<i>ehlersii</i>	Lengyel et al., 2005
	<i>innexi</i>	Lengyel et al., 2005
	<i>szentirmaii</i>	Lengyel et al., 2005
	<i>indica</i>	Somvanshi et al., 2006
	<i>hominickii</i>	Tailliez et al., 2006
	<i>kozodoii</i>	Tailliez et al., 2006
	<i>doucetiae</i>	Tailliez et al., 2006
	<i>cabanillasii</i>	Tailliez et al., 2006
	<i>griffiniae</i>	Tailliez et al., 2006
	<i>romanii</i>	Tailliez et al., 2006
	<i>miraniensis</i>	Tailliez et al., 2006
	<i>Stockiae</i>	Tailliez et al., 2006
	<i>koppenhoeferi</i>	Tailliez et al., 2006
<i>Photorhabdus</i>	<i>mauleonii</i>	Tailliez et al., 2006
	<i>vietnamensis</i>	Tailliez et al., 2009
	<i>luminescens</i> subsp. <i>akhurstii</i>	Fischer-Le Saux et al., 1999
	<i>luminescens</i> subsp. <i>kayaii</i>	Tailliez et al., 2010
	<i>luminescens</i> subsp. <i>caribbeanensis</i>	Tailliez et al., 2010
	<i>luminescens</i> subsp. <i>hainanensis</i>	Tailliez et al., 2010
	<i>luminescens</i> subsp. <i>kleinii</i>	An and Grewal 2011
	<i>temperata</i> subsp. <i>temperata</i>	Fischer-Le Saux et al., 1999

<i>temperata</i> subsp. <i>cinerea</i>	TÓth and Lakatos 2008
<i>temperata</i> subsp. <i>khanii</i>	Tailliez et al., 2010
<i>temperata</i> subsp. <i>thracensis</i>	Tailliez et al., 2010
<i>luminescens</i> subsp. <i>kayaii</i>	Tailliez et al., 2010
<i>temperata</i> subsp. <i>tasmaniensis</i>	Tailliez et al., 2010
<i>temperata</i> subsp. <i>stackebrandtii</i>	An and Grewal 2010
<i>asymbiotica</i> subsp. <i>asymbiotica</i>	Fischer-Le Saux et al., 1999
<i>asymbiotica</i> subsp. <i>australis</i>	Akhurst et al., 2004

8.5 Toxins of *Photorhabdus* bacteria

The complete genome of *Photorhabdus luminescens* encoded a large number of toxins, proteases and lipase (Duchaud et al., 2003). Toxins in *P. luminescens* subsp. *luminescens* TTO1 are classified into 4 groups; the toxin complexes (Tcs), the *Photorhabdus* insect related (Pir) proteins, the 'makes caterpillars floppy' (Mcf) toxins and the *Photorhabdus* virulence cassettes (PVC).

8.5.1 Toxin complexes (Tcs)

Toxin complexes (Tcs) complexes are encoded by the pathogenicity island I (PAI I) and have been identified as high molecular weight insecticidal toxin. Four complexes, Tca, Tcb, Tcc and Tcd have been found in different loci (Figure 2) (ffrench-Constant et al., 2007). Tca, Tcb, and Tcc were not similarities with other sequences in Genbank but *tccA* was similarity to *Salmonella* protein (SpvA), while TcaC was similarity to SpvB (Brown et al., 1998; ffrench-Constant et al., 1999). *tca* and *tcd* encode for orally active toxins of the insecticidal activity in *Manduca sexta* (Blackburn et al., 1998). Furthermore, Tca toxin against the Colorado potato beetle, *Leptinotarsa decemlineata* and the sweet potato whitefly *Bemisia tabaci* (Blackburn et al., 2005) and was shown to disrupt the insect midgut epithelium similar to the δ -endotoxins from *Bacillus thuringiensis* or *Bt* (Blackburn et al., 1998)

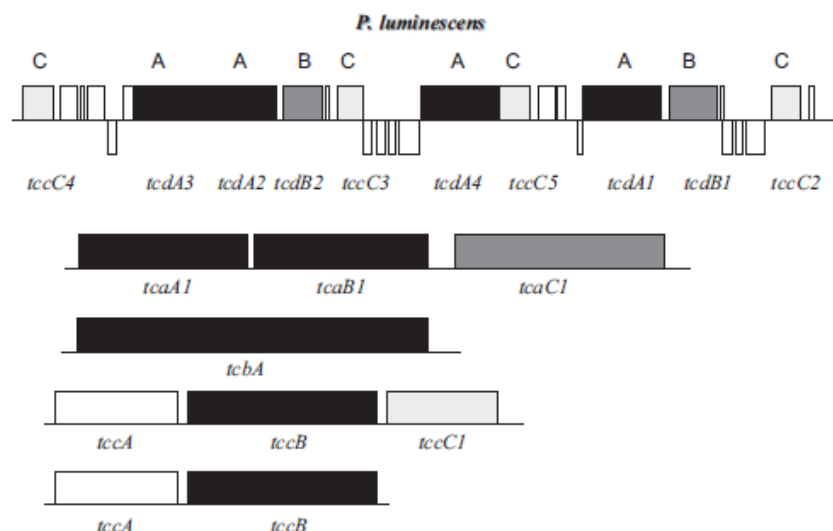


Figure 2 Classification of the tc genes in *P. luminescens* strain W14 (ffrench-Constant et al., 2007)

8.5.2 *Photorhabdus* Insect Related (Pir) Toxins

The '*Photorhabdus* insect related' (Pir) toxins are encoded by the *PirAB* genes, located at *plu4093-4092* (*pirA*) and *plu4437-4436* (*pirB*) loci of *P. luminescens* TT01 genome. Toxins from both loci were orally against the caterpillar pest *Pluteela xylostella* and toxin from *plu4093-4092* loci against a wide range of mosquito larvae (Duchaud et al., 2003). Knockout of *plu4092* in *P. luminescens* showed abolished toxicity to both caterpillars and mosquitoes (Duchaud et al., 2003). PirA and PirB protein were similarities to the δ -endotoxins of *B. thuringiensis* (Waterfield et al., 2005) and PirB was high homology with the N-terminal region of the pore-forming domain of the Cry2A insecticidal toxin and similarities with a developmentally regulated protein from the beetle *Leptinotarsa decemlineata*. *PirAB* from *P. luminescens* when expressed in *E. coli* had the ability to kill *Galleria mellonella* (Waterfield et al., 2005) PirAB toxin from *P. asymbiotica* when expressed in *E. coli* against *Aedes aegypti* and *Aedes albopictus* larvae (Ahantarig et al., 2009). The '*Photorhabdus* insect related' (Pir) are found in the same genomic location in 2 different strains of *Photorhabdus* (Figure 3).



Figure 3 Genomic organization of the *pirAB* operons in *P. luminescens* and *P. asymbiotica* (adapted from Ahantarig et al., 2009)

8.5.3 Makes Caterpillars Floppy toxins or Mcf

The 'makes caterpillars floppy' toxins 1 (Mcf1) and 2 (Mcf2) are encoded by PAI II (Waterfield et al., 2004). Mcf1 is longer than Mcf2 and in N-terminal domain of Mcf1 contains a Bcl2-homology 3-like domain (BH3 domain), it mimics BH3 domain proteins that are found in mitochondria and have pro-apototic actions. In the middle of Mcf1 has a Cytotoxin B-like motif and highly similar to *Clostridium* cytotoxin B (CdtB), involved in toxin translocation/ endocytosis (Hofmann et al., 1997), while the C-terminal domain of Mcf1 resembles the repeats-in toxin (RTX) like toxins of another bacterium (*Actinobacillus pleuropneumoniae*) (Dowling et al., 2004). In Mcf2, a RTX-like motif is not found in the C-terminus. N-terminus of Mcf2 has similar to a type III secreted protein of a plant pathogen (*Pseudomonas syringae*) but it lacks the BH3-like domain. Mcf2 has a region similar to CdtB, but it also possesses another domain, similar to HrmA protein (a protein secreted by a type III secretion system) in the N-terminus (Figure 4). Mcf1 and Mcf2 toxins, when expressed in *E. coli*, allow the bacterium to survive inside the insect and promote its death (Rodou et al., 2010).

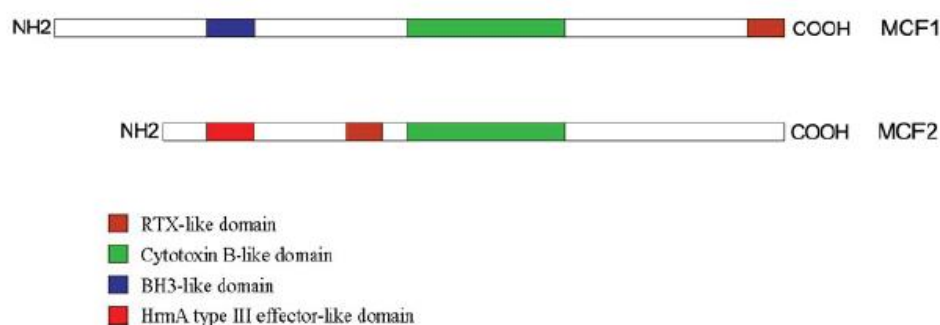


Figure 4 The domain structures of the Mcf proteins (Rodou et al., 2010)

8.5.4 *Photorhabdus* Virulence Cassettes (PVC)

PVC genes have sequence similarities to Mcf of *P. luminescens* or the toxin A of *C. difficile* (French-Constant et al., 2007). Comparison of the genomic organization of several PVCs shows that they have a conserved phage-like structure with a variable number of putative anti-insect effectors encoded at one end. Prophage-like loci are divided into two sections. First, the PVC element-like section containing an array of phage related genes. Second, the 'payload' region encodes different putative effectors or toxins (Figure 5). Electron microscopy of PVCs structure has similar to the antibacterial R-type pyocins, a type of bacteriocin. The protein products of the PVCs

have no direct antibacterial activity, but do destruct insect hemocytes. These proteins also show similarities to phage tail and base plate assembly proteins, fimbrial usher and proteins from other pathogenic bacteria. Furthermore, their effector proteins are located always downstream of the PVCs and are flanked by transposon sequences, indicative of a possible mechanism of insertion in the PVC or even their movement among different PVCs (Yang et al., 2006). Recombinant *E. coli* expressing PVC containing cosmids from *P. luminescens* have injectable insecticidal activity against larvae of wax moth (ffrench-Constant et al., 2007).

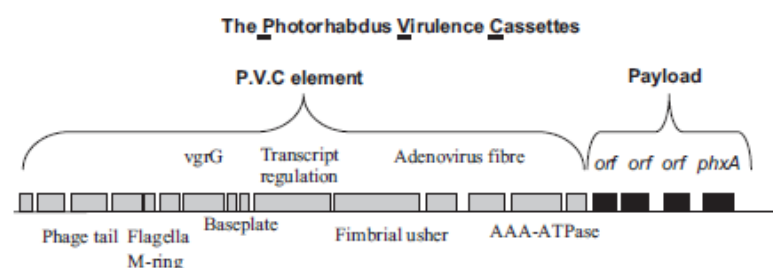


Figure 5 Genomic organization of the '*Photorhabdus Virulence Cassettes*' or PVCs

Objectives

To investigate the killing effect of whole cell suspension, cell-free supernatant, crude extract, bacterial cell pellet and recombinant pirA/pirB protein from *Xenorhabdus* and *Photorhabdus* bacteria from Thailand on *Aedes*, *Anopheles* and *Culex* mosquitoes larvae.

Research methodology

1. Whole cell suspension, cell-free supernatant and bacterial cell pellet and crude extract preparation

1.1 Bacteria culture

Xenorhabdus and *Photorhabdus* spp were subcultured on nutrient bromothymol blue triphenyltetrazolium chloride agar (NBTA) medium, consisting of 37 g nutrient agar, 25 mg bromothymol blue powder, 4 ml of 0.01 g/ml 2,3,5-triphenyltetrazolium chloride, and 1,000 ml distilled water. These plates will be sealed with parafilm and incubated at room temperature in the dark at 48 h (Bussaman et al., 2012).

1.2 Whole cell suspension preparation

Single colony of each bacterium was subcultured on 25 ml Luria-Bertani (LB) broth and incubated with shaking at 200 rpm at 28°C for 48 h in the dark. Whole cell extract was obtained from bacteria at 10⁸ colony forming unit per ml (CFU/ml) in a sterile 1 g/L peptone solution (Bussaman et al., 2012).

1.3 Cell-free supernatant preparation

The bacteria was cultured on 25 ml Luria-Bertani (LB) broth and incubated with shaking at 200 rpm at 28°C for 48 h in the dark. The cell-free supernatant preparation was prepared from bacteria at a concentration of 10⁸ CFU/ml. The culture was centrifuged at 12,000 rpm, 4°C for 5 min. The supernatant was filtered using 0.22 µm filter (Bussaman et al., 2012).

1.4 Bacterial cell pellet and Crude extract preparation

The pellet cell from 2.3 was divides into 2 parts. First part of cell pellet were kept to insecticidal activity and second part cell pellet was resuspended to 10 ml LB broth. The suspension was sonicated at 4°C for 5 min and centrifuged at 12,000 rpm at 4°C for 5 min. The supernatant was collected (Bussaman et al., 2012).

1.5 Recombinant pirA/pirB protein preparation

1.5.1 Bacteria culture and DNA extraction

Xenorhabdus sp. and *Photorhabdus* sp. were subcultured on NBTA and incubated at room temperature for 4 days. Single colony of each strain was inoculated

in 3 ml LB broth and incubated 37°C with shaking at 200 rpm for overnight. Three ml of overnight cultured was centrifuged and collected the pellet for DNA extraction. Genomic DNA extraction was used the Genomic DNA Mini Kit as recommended by the manufacturer (Geneaid Biotech Ltd., Taiwan).

1.5.2 Polymerase chain reaction and cloning of *pirA*/*pirB* gene

Primers and DNA amplification of *pirA* and *PirB* gene were modified from previously study (Waterfield et al, 2005). The primer sequence of PirA_F was ATCCATATGATGTCTAGAATAACCATTGTTG (underline was the position for *SphI*) and PirA_R was ATCGCATGCTTAAACCAAATTTGCCGTCAC (underline was the position for *SphI*). The primer sequence of PirB_F was CGAGCGGCCGCATGCATACAGAAAATGTTTTAG (underline was the position for *NotI*) and PirB_R was ATACAAAGCTTGCCGACATCAAAAGA (underline was the position for *HindIII*). PCR products were cloned into Expression vector pET32a and transformed the recombinant vector into *E. coli* (BL21).

The positive transformants were selected by spread on LB agar containing 100 µg/ml of ampicillin. The recombinant plasmid was purified by using Gel/PCR DNA Fragments Extraction Kit as recommended by the manufacturer (Geneaid Biotech Ltd., Taiwan) and checked by PCR. The recombination of the forward or reverse sequencing primer and a primer that hybridizes within target gene were used for PCR. .

Expression vector pET32a was transformed into *E. coli* strain LBL21 for used as a positive control and cell without vector was used as a negative control in gene expression step.

1.5.3 Expression of recombinant *pirAB* gene

Single colony of transformant and positive and negative control Expression vector pET32a and cells without plasmid vector were used as a positive and negative control, respectively) inoculated to 3 ml LB medium containing glucose and 100 µg/ml of ampicillin and incubated at 37°C with shaking 200 rpm for overnight. 100 hundred µl of the overnight cultured was added into 900 µl of LB medium containing 100 µg/ml of ampicillin and incubated at 37°C with shaking 200 rpm for 2 h and IPTG was added to a final concentration of 1 M to induce protein expression, 1 ml of cells was aliquot every 2, 4, 6 and 8h. Cell cultured was centrifuged at 12,000 rpm for 5 min and kept at -20°C. Supernatant and pellet cell were suspended in 80 µl of 1X SDS-

PAGE sample buffer. The cell suspension was boiled for 5 min and centrifuged at 12,000 rpm for 1 min. Ten μ l of sample was loaded on SDS-PAGE gel and electrophoresed. The polyacrylamide gel will be stained with Coomassie blue. The PirA and PirB protein bands were visualized and compared to prestained protein marker. The molecular weight of PirA and PirB protein should be 14.8 kDa and 46.2 kDa, respectively.

2. Insect bioassay

Mosquito larvae were tested for the insecticide activity with whole cell suspension, cell-free supernatant, crude extract and cell pellet of *Xenorhabdus* and *Photorhabdus* bacteria.

Twenty larvae of each mosquito genus (*Aedes*, *Anopheles* and *Culex*) were placed individually in a beaker containing 1,000 ml of dechlorinated tap water (pH 7.0) at 25°C and 75% relative humidity (Ahantari et al., 2009).

The toxicity of whole cell suspension, cell-free supernatant, crude cell extract and cell pellet were tested against mosquito larvae (*Aedes*, *Anopheles* and *Culex*) in triplicate experiments. pBAD plasmid and LB broth was used as negative control. The mortality rate of mosquito larvae of each genus was recorded daily for 7 days (Ahantari et al., 2009).

Result

1. *Xenorhabdus* and *Photorhabdus* were used for insecticide activity

A total of 20 isolates of *Xenorhabdus* and 15 isolates of *Photorhabdus* were used in this study (Table 3).

Table 3 List of *Xenorhabdus* and *Photorhabdus* bacteria for insecticide activity

Code	Bacteria
PB3.4	<i>Xenorhabdus stockiae</i>
PB7.2	<i>Xenorhabdus stockiae</i>
PB10.5	<i>Xenorhabdus stockiae</i>
PB57.4	<i>Xenorhabdus stockiae</i>
PB79.4	<i>Xenorhabdus stockiae</i>
KK3.1	<i>Xenorhabdus stockiae</i>
KK3.3	<i>Xenorhabdus stockiae</i>
NK6.3	<i>Xenorhabdus stockiae</i>
KJ12.5	<i>Xenorhabdus stockiae</i>
CP27.2	<i>Xenorhabdus stockiae</i>
PB39.3	Closely related to <i>Xenorhabdus stockiae</i>
PB63.1	Closely related to <i>Xenorhabdus stockiae</i>
CP37.1	Closely related to <i>Xenorhabdus stockiae</i>
SP6.1	Closely related to <i>Xenorhabdus stockiae</i>
KK7.4	Closely related to <i>Xenorhabdus stockiae</i>
CP54.1	Closely related to <i>Xenorhabdus stockiae</i>
CP59.1	Closely related to <i>Xenorhabdus stockiae</i>
PB62.2	Closely related to <i>Xenorhabdus stockiae</i>
PB62.4	<i>Xenorhabdus miranensis</i>
PP63.3	<i>Xenorhabdus miranensis</i>
PB45.5	<i>Photorhabdus luminescens</i>
PB67.2	<i>Photorhabdus luminescens</i>
PB58.2	<i>Photorhabdus luminescens</i>
SP7.3	<i>Photorhabdus luminescens</i>
CP47.1	<i>Photorhabdus luminescens</i>
CP49.5	<i>Photorhabdus luminescens</i>

KK1.3	<i>Photorhabdus luminescens</i> subsp. <i>hainaensis</i>
CP31.1	<i>Photorhabdus luminescens</i> subsp. <i>hainaensis</i>
CP31.2	<i>Photorhabdus luminescens</i> subsp. <i>hainaensis</i>
CP59.3	<i>Photorhabdus luminescens</i> subsp. <i>hainaensis</i>
CP59.2	<i>Photorhabdus luminescens</i> subsp. <i>hainaensis</i>
CP59.4	<i>Photorhabdus luminescens</i> subsp. <i>hainaensis</i>
NK2.5	<i>Photorhabdus luminescens</i> subsp. <i>akhurstii</i>
CP48.1	<i>Photorhabdus luminescens</i> subsp. <i>akhurstii</i>
CP25.2	<i>Photorhabdus luminescens</i> subsp. <i>laumondii</i>

2. Insecticide activity of whole cell suspension, cell free supernatant, crude cell extract and bacterial cell pellet from *Xenorhabdus* and *Photorhabdus* against larva stage 1-2 and 3-4 of *Aedes*

2.1 Insecticide activity of whole cell suspension of *Xenorhabdus* and *Photorhabdus* from against larva stage 1-2 and 3-4 of *Aedes*

Photorhabdus luminescens subsp. *hainaensis* (KK1.3) showed highest effectiveness on larva stage 1-2. Mortality rate was 60%, 80%, 80% and 80% after exposure to whole cell suspension of this bacterium for day 1, 2, 3 and 4, respectively (Table 4 and Figure 6). However, whole cell suspension of all bacteria showed lower effectiveness on larva stage 3-4 (Table 5 and Figure 7).

Table 4 Insecticidal activity of whole cell suspension from *Xenorhabdus* and *Photorhabdus* against larva stage 1-2 of *Aedes*

Bacteria code	% Mortality rate				
	Day 1	Day 2	Day 3	Day 4	Day 5
PB10.5	0%	0%	10%	15%	20%
PB57.4	0%	0%	0%	5%	15%
PB62.4	5%	15%	20%	30%	30%
PB62.2	5%	5%	5%	25%	35%
KK1.3	65%	80%	80%	80%	80%
NK2.5	0%	0%	0%	0%	0%
PB3.4	0%	0%	0%	0%	0%
PB6.3	0%	0%	0%	5%	5%
PB45.5	0%	0%	0%	5%	10%
Control (<i>E. coli</i>)	15%	20%	20%	25%	25%
Control (LB)	15%	15%	20%	20%	20%
Control (DW)	0%	5%	5%	5%	5%

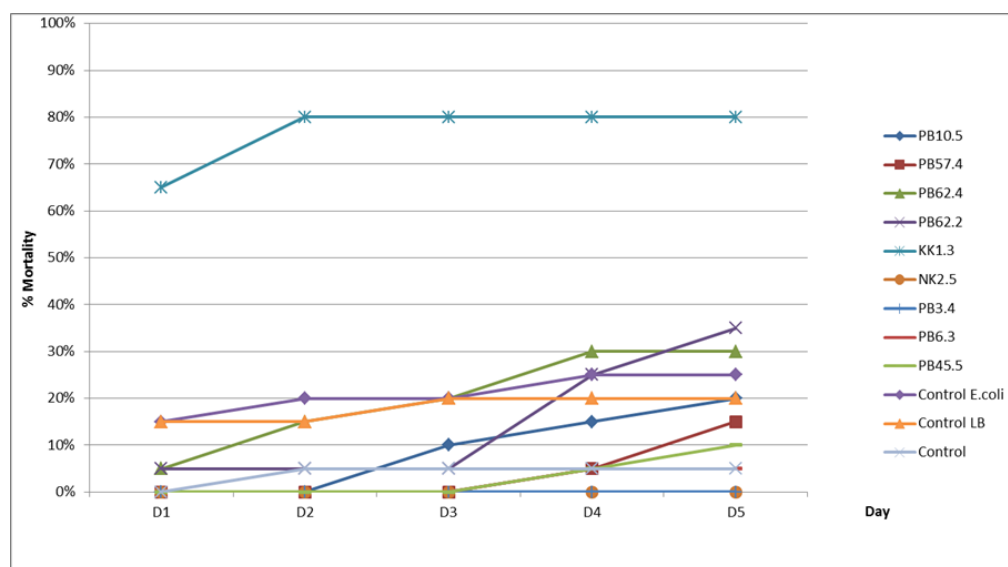


Figure 6 Mortality rate of larva stage 1-2 of *Aedes* after exposure to whole cell suspension of *Xenorhabdus* and *Photorhabdus*

Table 5 Insecticidal activity of whole cell suspension of *Xenorhabdus* และ *Photorhabdus* against larva stage 3-4 of *Aedes*

Bacteria code	% Mortality rate				
	Day 1	Day 2	Day 3	Day 4	Day 5
PB10.5	10%	10%	10%	10%	15%
PB57.4	0%	0%	5%	10%	15%
PB62.4	0%	0%	5%	5%	10%
PB62.2	0%	0%	5%	5%	10%
KK1.3	0%	0%	10%	15%	15%
NK2.5	0%	0%	5%	5%	10%
PB3.4	0%	0%	0%	0%	0%
PB6.3	0%	0%	0%	0%	5%
PB45.5	0%	0%	0%	5%	5%
Control (<i>E. coli</i>)	0%	0%	0%	5%	5%
Control (LB)	0%	0%	0%	0%	0%
Control (DW)	5%	5%	10%	10%	10%

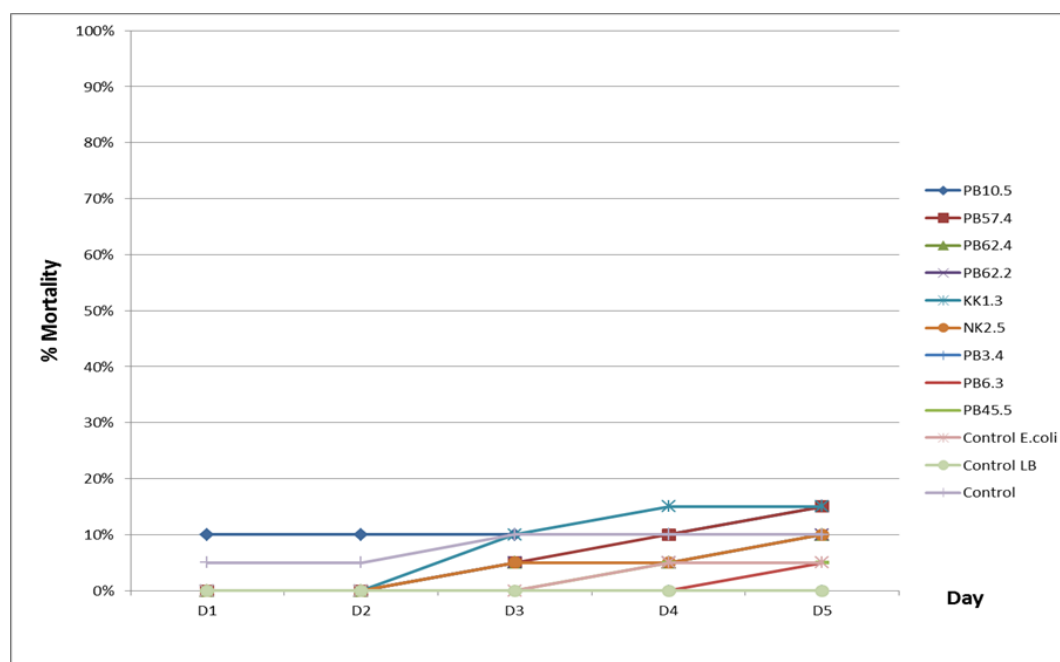


Figure 7 Mortality rate of larva stage 3-4 of *Aedes* after exposure to whole cell suspension of *Xenorhabdus* and *Photorhabdus*

2.2 Insecticide activity of cell free supernatant of *Xenorhabdus* and *Photorhabdus* against larva stage 1-2 and 3-4 of *Aedes*

The result showed cell free supernatant of *Photorhabdus luminescens* subsp. *hainaensis* (KK1.3) was highly effective to kill larva stage to 1-2. Mortality rate was 65% on 24 h after exposure to cell free supernatant of this bacterium and then mortality rate was increase to 70% on day 2, 3 and 4 (Table 6 and Figure 8). However, cell free supernatant of all bacteria showed lower effectiveness on larva stage 3-4 (Table 7 and Figure 9).

Table 6 Insecticidal activity of cell free supernatant of *Xenorhabdus* และ *Photorhabdus* against larva stage 1-2 of *Aedes*

Bacteria code	% Mortality rate				
	Day 1	Day 2	Day 3	Day 4	Day 5
PB10.5	10%	10%	20%	20%	25%
PB57.4	10%	10%	25%	35%	40%
PB62.4	0%	0%	5%	5%	5%
PB62.2	0%	0%	15%	5%	0%
KK1.3	65%	70%	70%	70%	70%
NK2.5	0%	0%	0%	0%	0%
PB3.4	10%	20%	20%	20%	20%
PB6.3	0%	0%	0%	0%	0%
PB45.5	0%	0%	0%	0%	0%
Control (<i>E. coli</i>)	25%	25%	25%	25%	25%
Control (LB)	15%	15%	20%	20%	20%
Control (DW)	0%	5%	5%	5%	5%

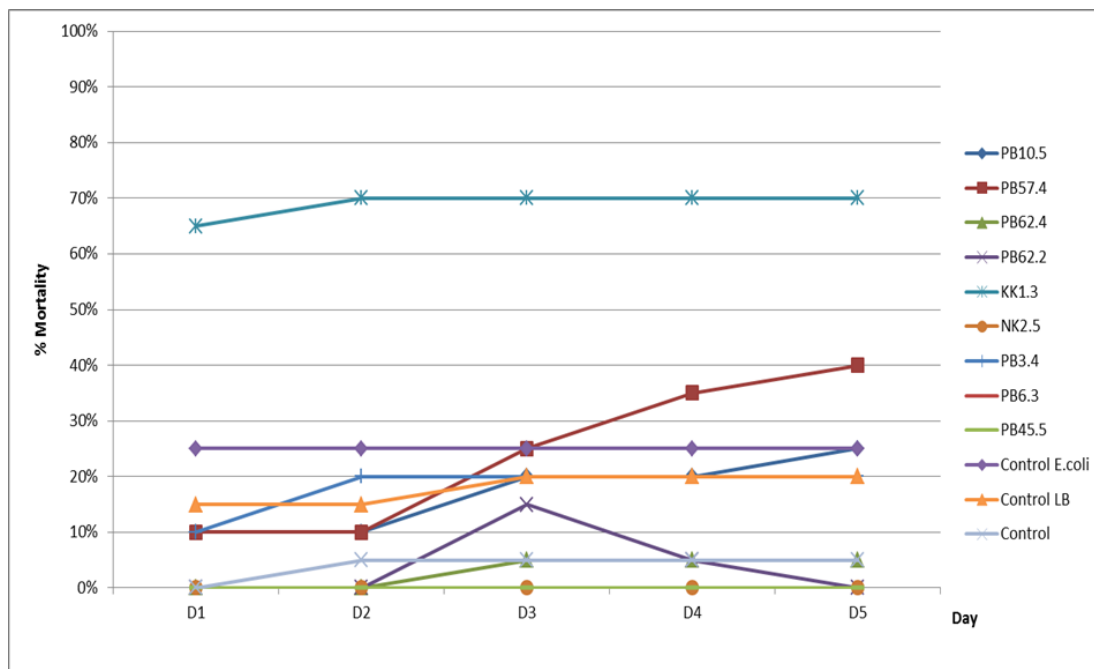


Figure 8 Mortality rate of larva stage 1-2 of *Aedes* after exposure to cell free supernatant of *Xenorhabdus* and *Photorhabdus*

Table 7 Insecticidal activity of cell free supernatant of *Xenorhabdus* and *Photorhabdus* against larva stage 3-4 of *Aedes*

Bacteria code	% Mortality rate				
	Day 1	Day 2	Day 3	Day 4	Day 5
PB10.5	0%	0%	5%	5%	5%
PB57.4	0%	0%	0%	5%	10%
PB62.4	5%	5%	5%	10%	10%
PB62.2	0%	0%	10%	10%	10%
KK1.3	0%	0%	0%	0%	0%
NK2.5	0%	0%	0%	0%	5%
PB3.4	5%	5%	5%	5%	20%
PB6.3	0%	0%	0%	0%	0%
PB45.5	0%	0%	0%	0%	0%
Control (<i>E. coli</i>)	0%	0%	5%	5%	5%
Control (LB)	0%	0%	0%	0%	0%
Control (DW)	5%	5%	10%	10%	10%

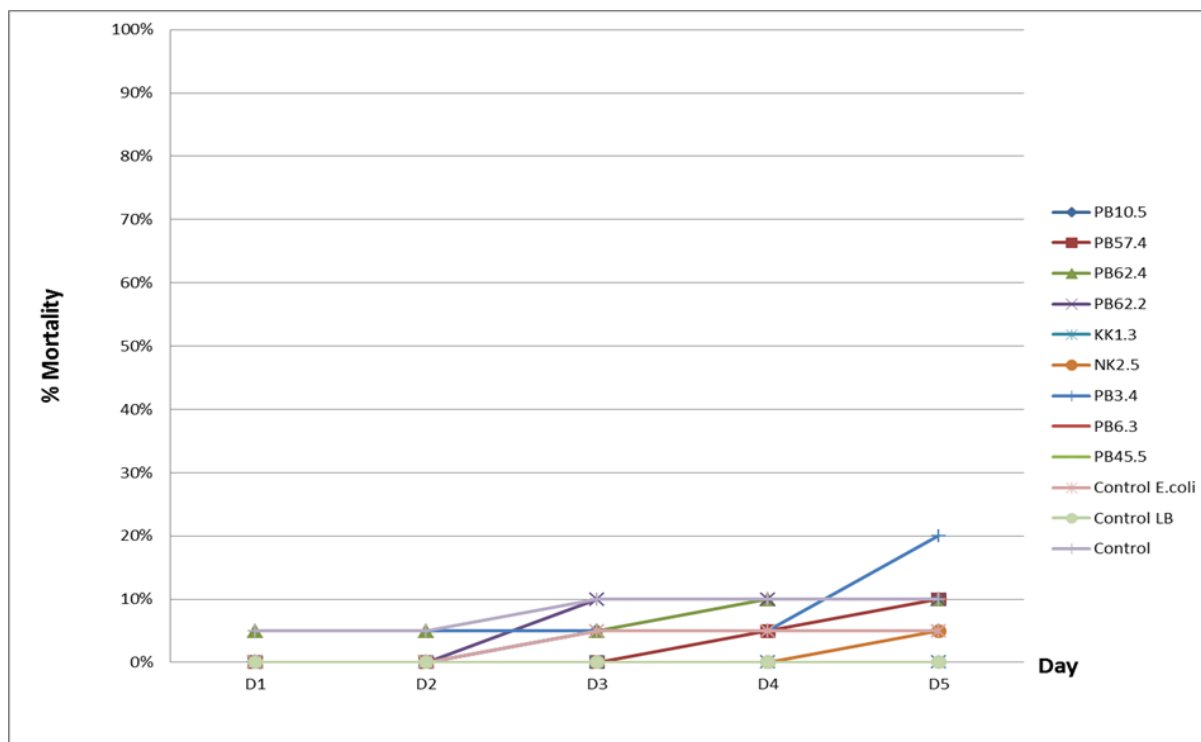


Figure 9 Mortality rate of larva stage 3-4 of *Aedes* after exposure to cell free supernatant of *Xenorhabdus* and *Photorhabdus*

2.3 Insecticide activity of crude cell extract of *Xenorhabdus* and *Photorhabdus* against larva stage 1-2 and 3-4 of *Aedes*

Mortality rate of larva stage 1-2 and 3-4 after exposure to crude cell extract showed lower when compared to whole cell suspension and cell free supernatant (Table 8, 9 and Figure 10, 11).

Table 8 Insecticidal activity of crude cell extract of *Xenorhabdus* and *Photorhabdus* against larva stage 1-2 of *Aedes*

Bacteria code	% Mortality rate				
	Day 1	Day 2	Day 3	Day 4	Day 5
PB10.5	0%	20.0%	25%	40.0%	40%
PB57.4	10%	10.0%	20%	30.0%	30%
PB62.4	25%	30.0%	30%	40.0%	40%
PB62.2	5%	5.0%	10%	15.0%	20%
KK1.3	5%	15.0%	15%	15.0%	20%
NK2.5	5%	5.0%	5%	10.0%	15%
PB3.4	0%	0.0%	0%	15.0%	15%
PB6.3	5%	10.0%	10%	10.0%	10%
PB45.5	25%	30.0%	35%	35.0%	35%
Control (<i>E. coli</i>)	25%	30.0%	45%	45.0%	45%
Control (PBS)	30%	40.0%	45%	45.0%	45%
Control (DW)	0%	15.0%	15%	20.0%	20%

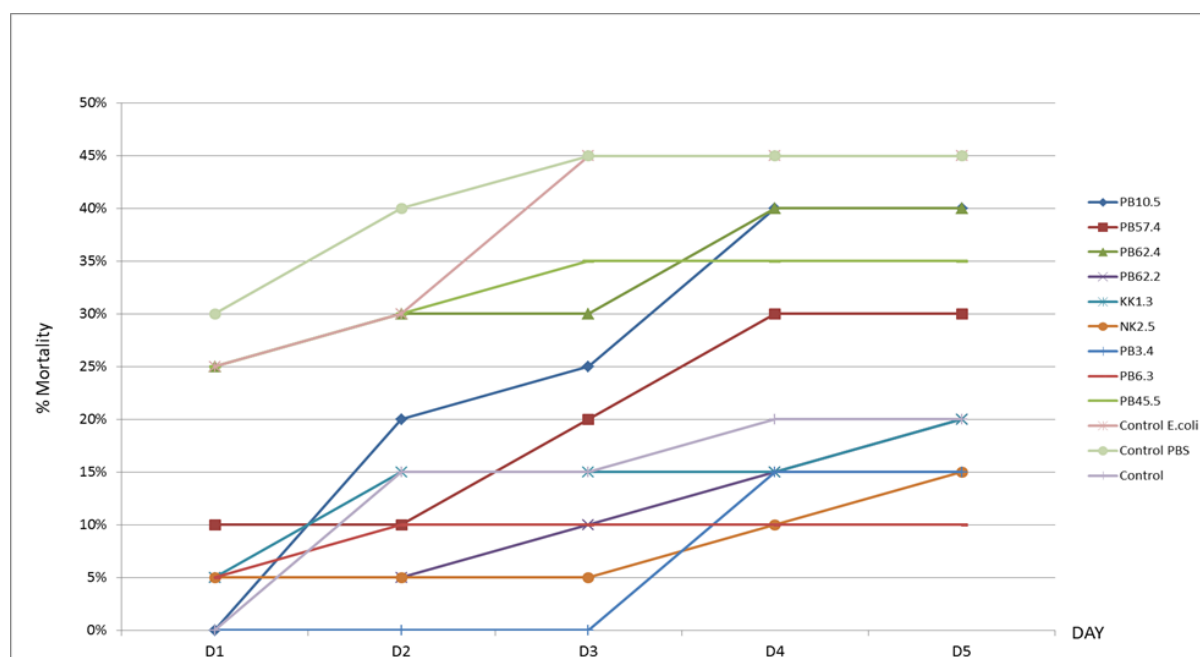


Figure 10 Mortality rate of larva stage 1-2 of *Aedes* after exposure to crude cell extract of *Xenorhabdus* and *Photorhabdus*

Table 9 Insecticidal activity of crude cell extract of *Xenorhabdus* and *Photorhabdus* against larva stage 3-4 of *Aedes*

Bacteria code	% Mortality rate				
	Day 1	Day 2	Day 3	Day 4	Day 5
PB10.5	0%	0%	0%	0%	5%
PB57.4	0%	0%	0%	5%	20%
PB62.4	0%	0%	0%	5%	5%
PB62.2	0%	0%	0%	0%	0%
KK1.3	0%	0%	0%	0%	5%
NK2.5	5%	5%	10%	10%	10%
PB3.4	0%	0%	0%	5%	10%
PB6.3	0%	0%	0%	5%	5%
PB45.5	0%	0%	0%	5%	10%
Control (<i>E.coli</i>)	0%	0%	10%	10%	10%
Control (PBS)	5%	5%	5%	10%	10%
Control (DW)	5%	5%	10%	10%	10%

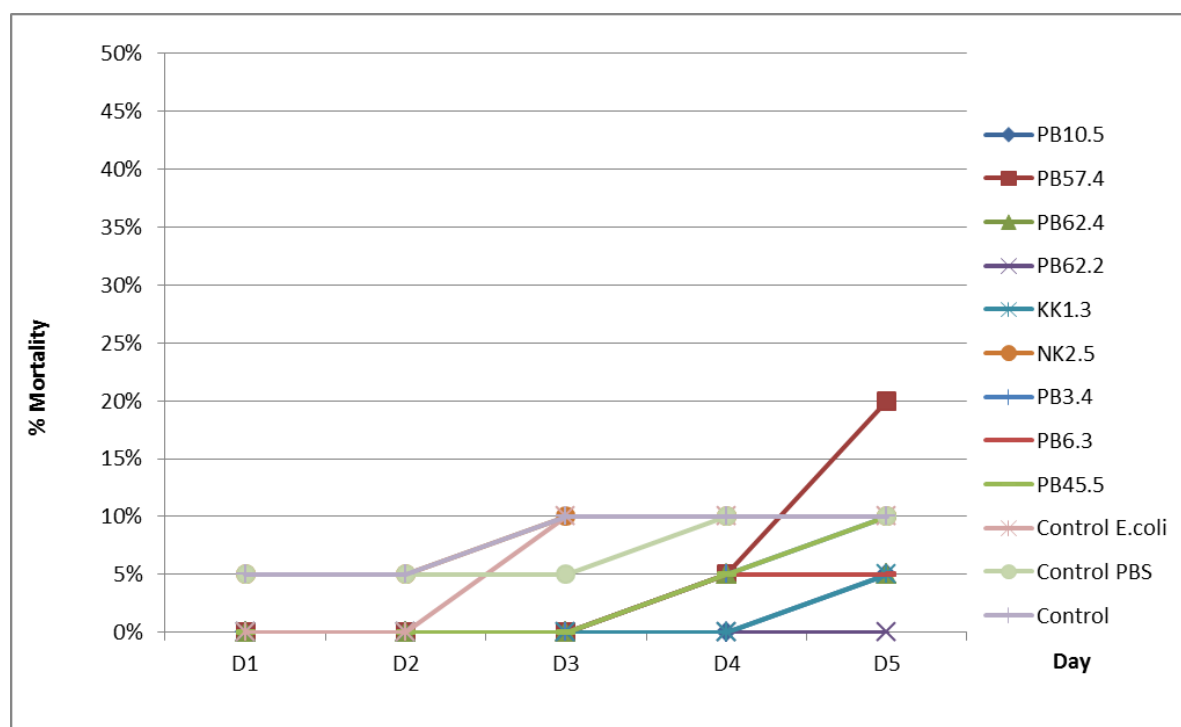


Figure 11 Mortality rate of larva stage 3-4 of *Aedes* after exposure to crude cell extract of *Xenorhabdus* and *Photorhabdus*

2.4 Insecticide activity of bacterial cell pellet of *Xenorhabdus* and *Photorhabdus* against larva stage 1-2 and 3-4 of *Aedes*

Photorhabdus luminescens subsp. *hainaensis* (KK1.3) and *Photorhabdus luminescens* subsp. *akhurstii* (NK2.5) showed highest effectiveness on larva stage 1-2. Mortality rate was found 100% on 48 h of both bacteria. (Table 10 and Figure 12). Furthermore, bacterial cell pellet of *Photorhabdus luminescens* subsp. *hainaensis* (KK1.3) was highly effective to kill larva stage to 3-4 (Table 11 and Figure 13).

Table 10 Insecticidal activity of bacterial cell pellet of *Xenorhabdus* และ *Photorhabdus* against larva stage 1-2 of *Aedes*

Bacteria code	% Mortality rate				
	Day 1	Day 2	Day 3	Day 4	Day 5
PB10.5	0%	20.0%	25%	40.0%	40%
PB57.4	10%	10.0%	20%	30.0%	30%
PB62.4	25%	30.0%	30%	40.0%	40%
PB62.2	5%	5.0%	10%	15.0%	20%
KK1.3	5%	15.0%	15%	15.0%	20%
NK2.5	5%	5.0%	5%	10.0%	15%
PB3.4	0%	0.0%	0%	15.0%	15%
PB6.3	5%	10.0%	10%	10.0%	10%
PB45.5	25%	30.0%	35%	35.0%	35%
Control (<i>E. coli</i>)	25%	30.0%	45%	45.0%	45%
Control (PBS)	30%	40.0%	45%	45.0%	45%
Control (DW)	0%	15.0%	15%	20.0%	20%

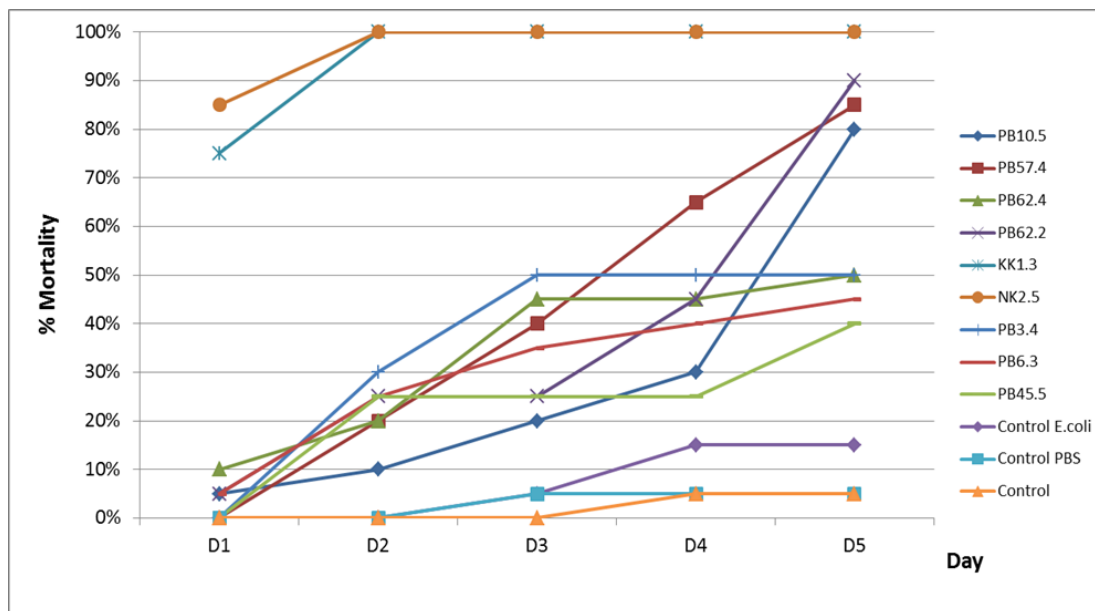


Figure 12 Mortality rate of larva stage 1-2 of *Aedes* after exposure to bacterial cell pellet of *Xenorhabdus* and *Photorhabdus*

Table 11 Insecticidal activity of bacterial cell pellet crude cell extract of *Xenorhabdus* and *Photorhabdus* against larva stage 3-4 of *Aedes*

Bacteria code	% Mortality rate				
	Day 1	Day 2	Day 3	Day 4	Day 5
PB10.5	0%	0%	5%	15%	15%
PB57.4	5%	5%	5%	25%	25%
PB62.4	0%	0%	0%	5%	5%
PB62.2	5%	5%	5%	10%	15%
KK1.3	75%	90%	90%	90%	90%
NK2.5	0%	0%	0%	0%	0%
PB3.4	0%	0%	0%	0%	5%
PB6.3	0%	0%	0%	0%	0%
PB45.5	0%	0%	0%	0%	0%
Control (<i>E. coli</i>)	25%	40%	40%	40%	40%
Control (PBS)	0%	10%	15%	15%	25%
Control (DW)	15%	15%	20%	20%	20%

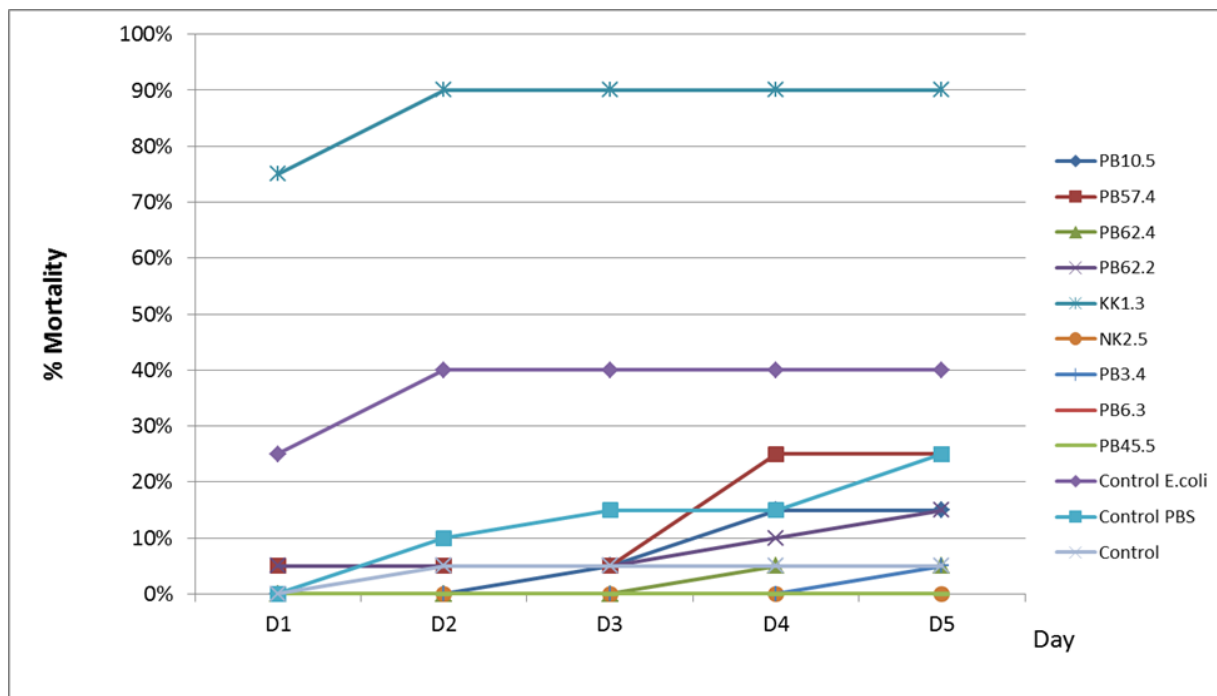


Figure 13 Mortality rate of larva stage 3-4 of *Aedes* after exposure to bacterial cell pellet of *Xenorhabdus* and *Photorhabdus*

3. Insecticide activity of whole cell suspension, cell free supernatant, crude cell extract and bacterial cell pellet from *Xenorhabdus* and *Photorhabdus* against larva stage 1-2 and 3-4 of *Culex*

3.1 Insecticide activity of whole cell suspension of *Xenorhabdus* and *Photorhabdus* from against larva stage 1-2 and 3-4 of *Culex*

Whole cell suspension of *Xenorhabdus miranensis* (PB62.2), *Xenorhabdus miranensis* (PB62.4), *Xenorhabdus stockiae* (PB57.4) and *Photorhabdus luminescens* subsp. *hainaensis* (KK1.3) showed highly effective to kill larva stage 1-2 of *Culex* (Table 12 and 13 and Figure 13 and 14).

Table 12 Insecticidal activity of whole cell suspension of *Xenorhabdus* และ *Photorhabdus* against larva stage 1-2 of *Culex*

Bacteria code	% Mortality rate				
	Day 1	Day 2	Day 3	Day 4	Day 5
PB10.5	0%	0%	0%	5%	10%
PB57.4	0%	25%	65%	75%	80%
PB62.4	0%	40%	70%	85%	95%
PB62.2	0%	70%	100%	100%	100%
PB6.3	5%	5%	5%	5%	15%
PB3.4	0%	0%	5%	5%	5%
NK2.5	0%	30%	30%	30%	35%
KK1.3	5%	80%	80%	80%	80%
PB45.5	0%	5%	5%	5%	10%
Control (<i>E.coli</i>)	5%	10%	10%	15%	15%
Control (LB)	5%	10%	20%	20%	20%
Control (DW)	0%	5%	15%	20%	50%

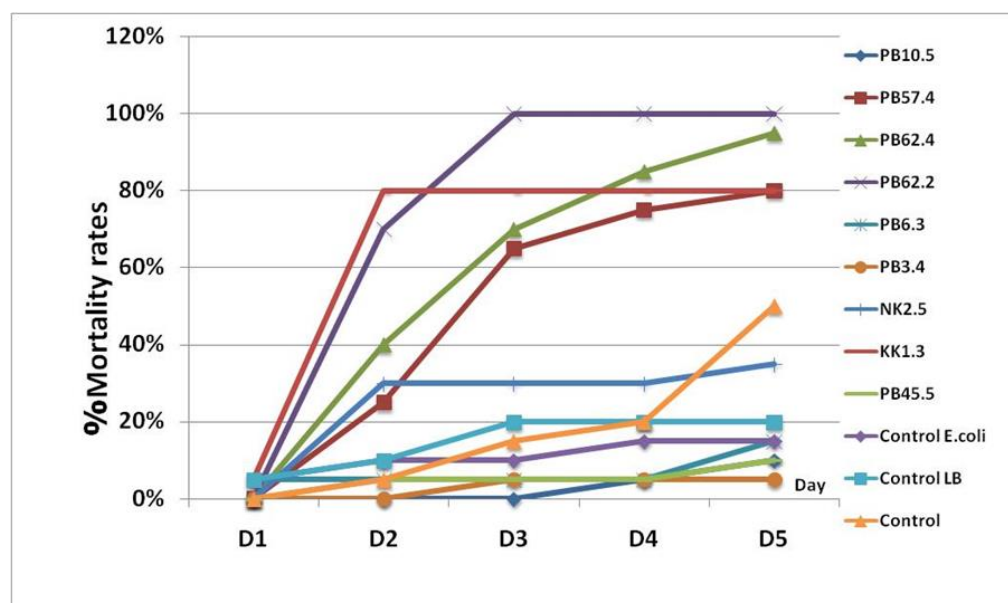


Figure 13 Mortality rate of larva stage 1-2 of *Culex* after exposure to whole cell suspension of *Xenorhabdus* and *Photorhabdus*

Table 13 Insecticidal activity of whole cell suspension of *Xenorhabdus* และ *Photorhabdus* against larva stage 3-4 of *Culex*

Bacteria code	% Mortality rate				
	Day 1	Day 2	Day 3	Day 4	Day 5
PB10.5	5%	5%	10%	20%	50%
PB57.4	10%	15%	15%	20%	75%
PB62.4	20%	20%	25%	40%	75%
PB62.2	5%	15%	15%	20%	90%
PB6.3	0%	5%	5%	15%	60%
PB3.4	0%	5%	5%	10%	50%
NK2.5	15%	65%	65%	65%	75%
KK1.3	5%	65%	65%	65%	75%
PB45.5	0%	0%	5%	15%	60%
Control (<i>E.coli</i>)	0%	0%	5%	15%	45%
Control (LB)	0%	0%	20%	20%	60%
Control (DW)	15%	25%	30%	40%	80%

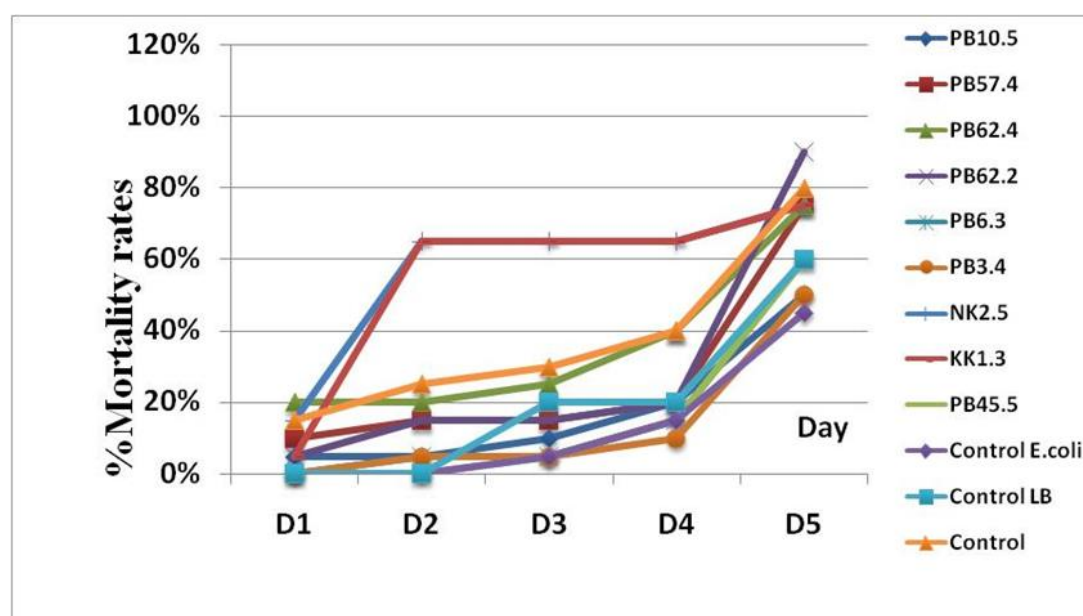


Figure 14 Mortality rate of larva stage 3-4 of *Culex* after exposure to whole cell suspension of *Xenorhabdus* and *Photorhabdus*

3.2 Insecticide activity of cell free supernatant of *Xenorhabdus* and *Photorhabdus* from against larva stage 1-2 and 3-4 of *Culex*

Cell free supernatant of *Xenorhabdus miranensis* (PB62.2), *Xenorhabdus miranensis* (PB62.4) *Xenorhabdus stockiae* (PB57.4 และ PB10.5)) and *Xenorhabdus miranensis* (PB62.2) showed highly effective to kill larva stage 1-2 of *Culex*, especially, *Xenorhabdus miranensis* (PB62.2) and *Xenorhabdus stockiae* (PB57.4) showed highest effectiveness on larva stage 1-2 and 3-4 of *Culex*, respectively (Table 14 and 15 and Figure 15 and 16).

Table 14 Insecticidal activity of cell free supernatant of *Xenorhabdus* และ *Photorhabdus* against larva stage 1-2 of *Culex*

Bacteria code	% Mortality rate				
	Day 1	Day 2	Day 3	Day 4	Day 5
PB10.5	0%	60%	60%	60%	75%
PB57.4	0%	5%	55%	60%	75%
PB62.4	0%	55%	90%	90%	100%
PB62.2	0%	70%	100%	100%	100%
PB6.3	0%	0%	5%	5%	5%
PB3.4	0%	0%	0%	0%	0%
NK2.5	0%	10%	20%	20%	20%
KK1.3	0%	5%	15%	15%	20%
PB45.5	0%	0%	10%	10%	10%
Control (<i>E.coli</i>)	0%	0%	0%	0%	0%
Control (LB)	5%	10%	20%	20%	20%
Control (DW)	0%	5%	15%	20%	50%

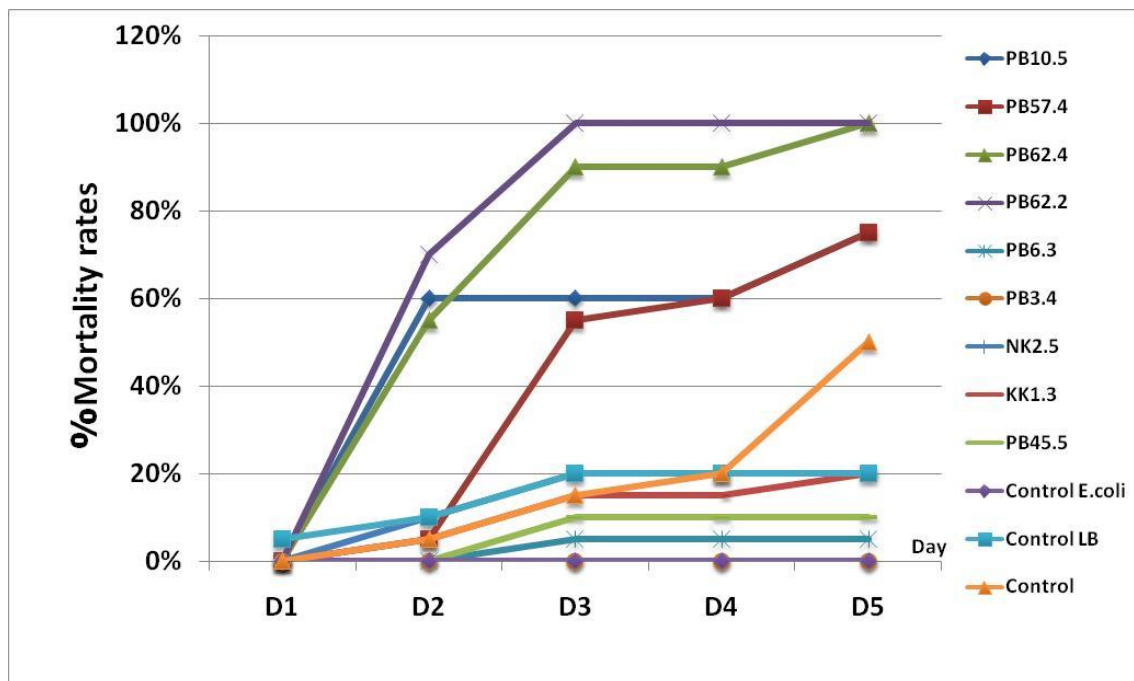


Figure 15 Mortality rate of larva stage 1-2 of *Culex* after exposure to cell free supernatant of *Xenorhabdus* and *Photorhabdus*

Table 15 Insecticidal activity of cell free supernatant of *Xenorhabdus* and *Photorhabdus* against larva stage 3-4 of *Culex*

Bacteria code	% Mortality rate				
	Day 1	Day 2	Day 3	Day 4	Day 5
PB10.5	0%	0%	5%	20%	80%
PB57.4	5%	5%	5%	25%	95%
PB62.4	5%	10%	10%	15%	50%
PB62.2	0%	0%	0%	10%	80%
PB6.3	5%	5%	5%	10%	50%
PB3.4	0%	0%	0%	5%	60%
NK2.5	10%	10%	20%	25%	45%
KK1.3	5%	10%	10%	25%	60%
PB45.5	0%	0%	0%	5%	40%
Control (<i>E.coli</i>)	0%	0%	0%	10%	50%
Control (LB)	0%	0%	20%	20%	60%
Control (DW)	15%	25%	30%	40%	80%

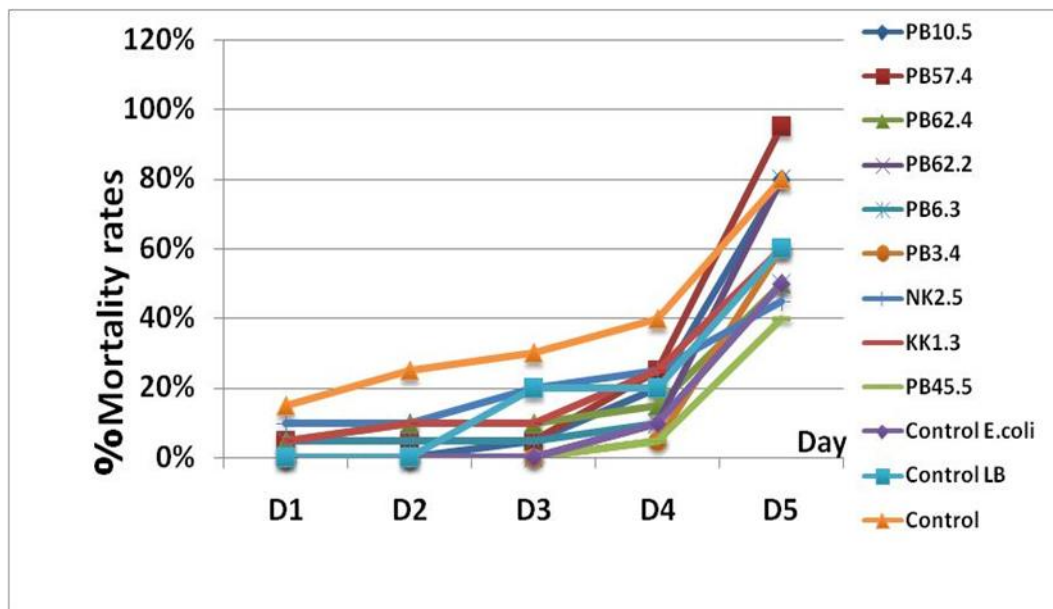


Figure 16 Mortality rate of larva stage 3-4 of *Culex* after exposure to cell free supernatant of *Xenorhabdus* and *Photorhabdus*

3.3 Insecticide activity of crude cell extract of *Xenorhabdus* and *Photorhabdus* from against larva stage 1-2 and 3-4 of *Culex*

Mortality rate of larva stage 3-4 of *Culex* was 100% on day 2 after exposure to crude cell extract of *Xenorhabdus stockiae* (PB57.4) and *Photorhabdus luminescens* subsp. *akhurstii* (NK2.5) (Table 15 and Figure 17). However, the mortality rate of larva stage 1-2 of *Culex* after exposure to crude cell extract of *Xenorhabdus* and *Photorhabdus* and control were not different (Table 16 and Figure 18).

Table 15 Insecticidal activity of crude cell extract of *Xenorhabdus* and *Photorhabdus* against larva stage 1-2 of *Culex*

Bacteria code	% Mortality rate				
	Day 1	Day 2	Day 3	Day 4	Day 5
PB10.5	0%	15%	20%	20%	20%
PB57.4	0%	0%	5%	10%	10%
PB62.4	0%	10%	15%	20%	20%
PB62.2	0%	0%	0%	0%	0%
PB6.3	0%	0%	0%	0%	0%
PB3.4	0%	0%	0%	5%	10%
NK2.5	0%	5%	15%	15%	15%
KK1.3	5%	5%	5%	10%	10%
PB45.5	0%	5%	10%	15%	20%
Control (<i>E.coli</i>)	5%	20%	25%	30%	30%
Control (LB)	5%	10%	25%	35%	40%
Control (DW)	0%	5%	15%	20%	50%

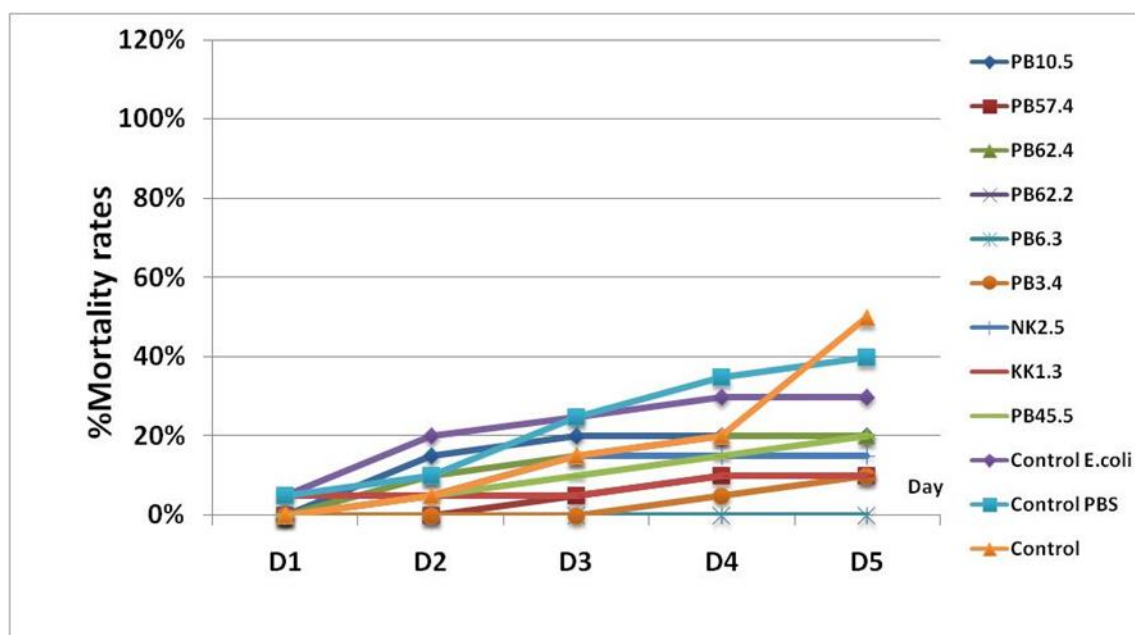


Figure 17 Mortality rate of larva stage 1-2 of *Culex* after exposure to crude cell extract of *Xenorhabdus* and *Photorhabdus*

Table 16 Insecticidal activity of crude cell extract of *Xenorhabdus* and *Photorhabdus* against larva stage 3-4 of *Culex*

Bacteria code	% Mortality rate				
	Day 1	Day 2	Day 3	Day 4	Day 5
PB10.5	5%	10%	10%	10%	20%
PB57.4	0%	5%	5%	5%	5%
PB62.4	0%	0%	5%	20%	25%
PB62.2	5%	15%	15%	15%	20%
PB6.3	0%	0%	0%	0%	35%
PB3.4	0%	0%	0%	0%	5%
NK2.5	60%	100%	100%	100%	100%
KK1.3	55%	95%	100%	100%	100%
PB45.5	5%	10%	10%	15%	85%
Control (<i>E.coli</i>)	10%	15%	20%	25%	50%
Control (LB)	0%	0%	0%	10%	90%
Control (DW)	15%	25%	30%	40%	80%

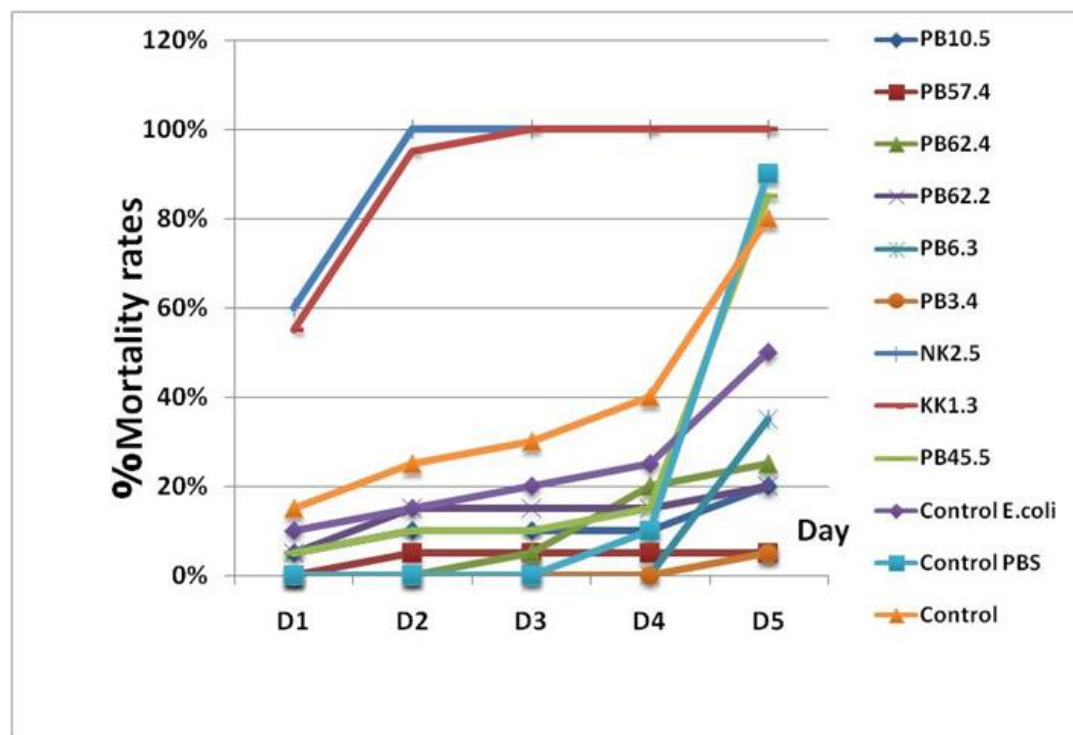


Figure 18 Mortality rate of larva stage 3-4 of *Culex* after exposure to crude cell extract of *Xenorhabdus* and *Photorhabdus*

3.4 Insecticide activity of bacterial cell pellet of *Xenorhabdus* and *Photorhabdus* from against larva stage 1-2 and 3-4 of *Culex*

Mortality rate of larva stage 3-4 of *Culex* was 100% on day 2 after exposure to crude cell extract of *Xenorhabdus stockiae* (PB57.4) and *Photorhabdus luminescens* subsp. *akhurstii* (NK2.5) (Table 17 and Figure 19). However, the mortality rate of larva stage 1-2 of *Culex* after exposure to crude cell extract of *Xenorhabdus* and *Photorhabdus* and control were not different (Table 18 and Figure 20).

Bacterial cell pellet of *Photorhabdus luminescens* subsp. *hainaensis* (KK1.3) and *Photorhabdus luminescens* subsp. *hainaensis* (KK1.3) showed highest effectiveness on larva stage 1-2 and 3-4 of *Culex*, respectively (Table 19, 20 and Figure 21, 22).

Table 17 Insecticidal activity of bacterial cell pellet of *Xenorhabdus* and *Photorhabdus* against larva stage 1-2 of *Culex*

Bacteria code	% Mortality rate				
	Day 1	Day 2	Day 3	Day 4	Day 5
PB10.5	5%	10%	15%	15%	15%
PB57.4	0%	5%	30%	40%	60%
PB62.4	5%	5%	70%	85%	95%
PB62.2	5%	25%	65%	65%	65%
PB6.3	0%	0%	5%	5%	5%
PB3.4	0%	65%	70%	70%	70%
NK2.5	0%	70%	80%	80%	80%
KK1.3	5%	95%	100%	100%	100%
PB45.5	5%	10%	15%	15%	15%
Control (<i>E.coli</i>)	0%	0%	5%	5%	5%
Control (PBS)	0%	0%	0%	20%	95%
Control (DW)	0%	5%	15%	20%	50%

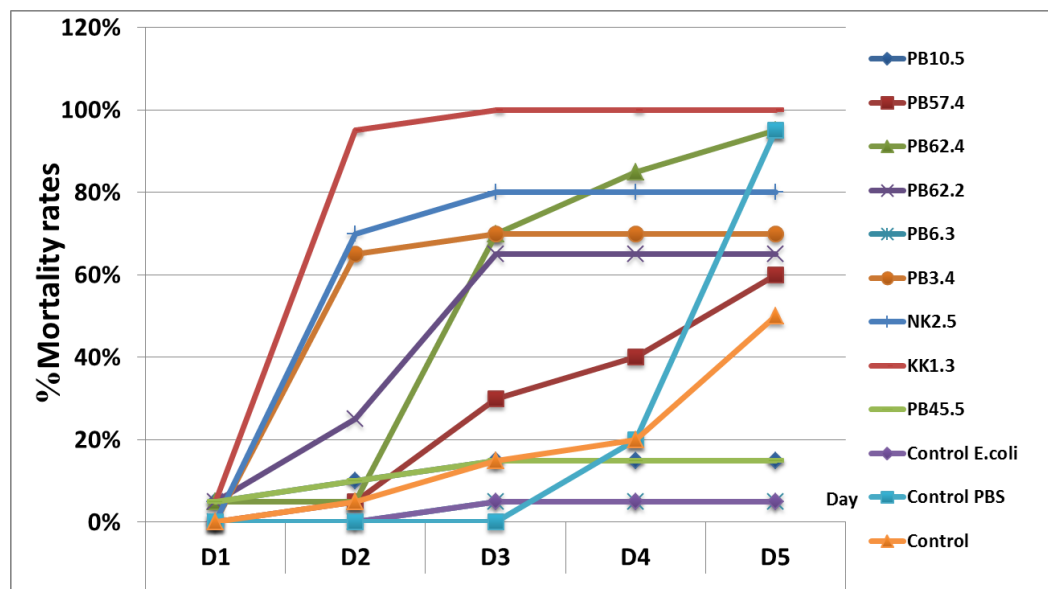


Figure 19 Mortality rate of larva stage 1-2 of *Culex* after exposure to bacterial cell pellet of *Xenorhabdus* and *Photorhabdus*

Table 18 Insecticidal activity of bacterial cell pellet of *Xenorhabdus* and *Photorhabdus* against larva stage 3-4 of *Culex*

Bacteria code	% Mortality rate				
	Day 1	Day 2	Day 3	Day 4	Day 5
PB10.5	5%	10%	10%	10%	20%
PB57.4	0%	5%	5%	5%	5%
PB62.4	0%	0%	5%	20%	25%
PB62.2	5%	15%	15%	15%	20%
PB6.3	0%	0%	0%	0%	35%
PB3.4	0%	0%	0%	0%	5%
NK2.5	60%	100%	100%	100%	100%
KK1.3	55%	95%	100%	100%	100%
PB45.5	5%	10%	10%	15%	85%
Control (<i>E.coli</i>)	10%	15%	20%	25%	50%
Control (PBS)	0%	0%	0%	10%	90%
Control (DW)	15%	25%	30%	40%	80%

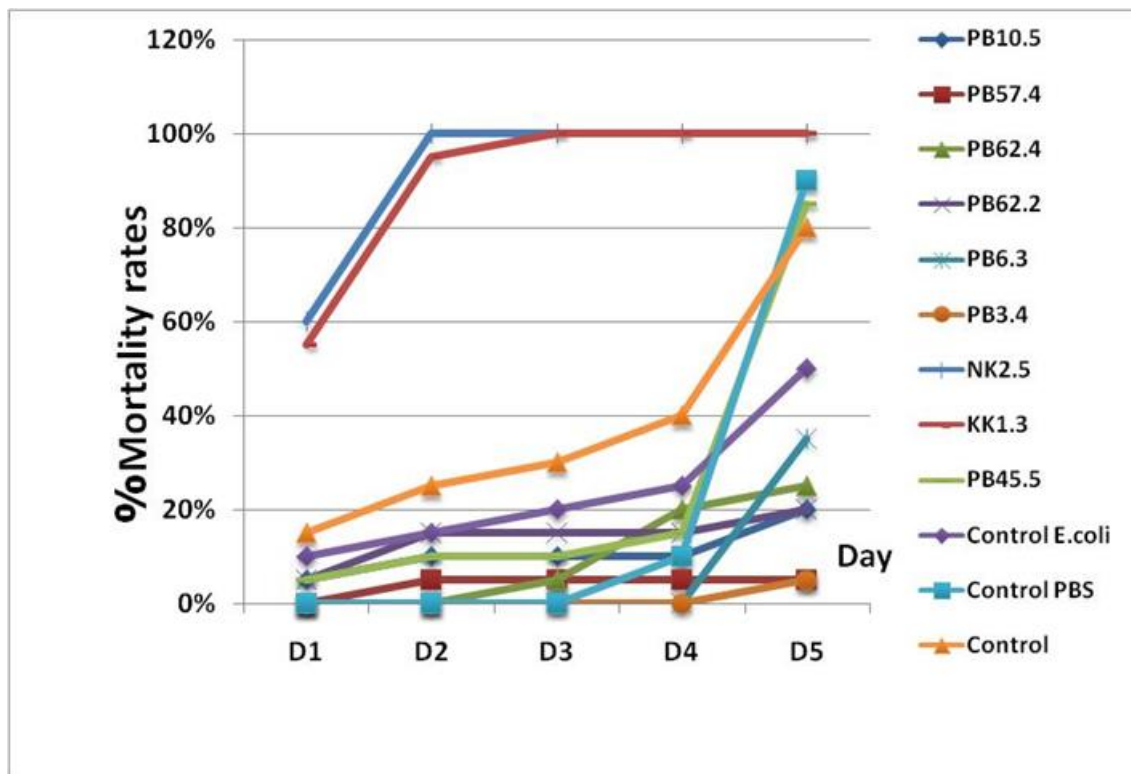


Figure 20 Mortality rate of larva stage 3-4 of *Culex* after exposure to bacterial cell pellet of *Xenorhabdus* and *Photorhabdus*

4 Insecticide activity of bacterial cell pellet from *Xenorhabdus* and *Photorhabdus* against larva stage 2 of *Aedes*, *Culex* and *Anopheles*

4.1 Insecticide activity of bacterial cell pellet of *Xenorhabdus* and *Photorhabdus* from against larva stage 2 of *Aedes*

Bacterial cell pellet of *Photorhabdus luminescens* subsp. *hainaensis* (CP31.1) and closely related to *Xenorhabdus stockiae* (CP59.4) showed effective to kill larva stage of *Aedes* but the mortality rate less than 50% (Table 21 and Figure 23).

Table 21 Insecticidal activity of bacterial cell pellet of *Xenorhabdus* and *Photorhabdus* against larva stage 2 of *Aedes*

Bacteria code	% Mortality rate			
	Day 1	Day 2	Day 3	Day 4
PB58.2	0%	0%	7%	7%
SP7.3	0%	0%	3%	7%
CP59.4	0%	27%	27%	33%
CP48.1	0%	0%	0%	7%
CP59.3	3%	3%	3%	13%
CP47.1	3%	3%	3%	13%
CP49.5	0%	0%	3%	7%
CP59.2	0%	0%	0%	13%
CP31.1	0%	0%	0%	13%
CP31.2	0%	33%	33%	33%
CP25.2	0%	13%	20%	20%
PB63.3	0%	0%	3%	2%
PB10.5	0%	3%	3%	3%
PB7.2	0%	10%	13%	13%
SP6.1	0%	13%	17%	17%
PB79.4	0%	10%	13%	13%
KK6.3	3%	17%	23%	23%
PB62.4	3%	10%	10%	10%
PB57.4	0%	10%	10%	10%
KK3.3	0%	3%	3%	3%
PB61.2	0%	3%	3%	3%
PB39.3	0%	17%	17%	17%
PB63.1	0%	17%	17%	17%
KJ12.5	0%	10%	10%	13%
KK7.4	3%	3%	3%	7%
KK3.1	7%	7%	10%	10%
CP27.2	0%	20%	20%	20%
CP37.1	0%	0%	3%	3%
CP54.1	0%	10%	10%	10%

CP59.1	0%	0%	0%	0%
Control (<i>E.coli</i>)	0%	0%	0%	0%
Control (DW)	0%	0%	0%	0%

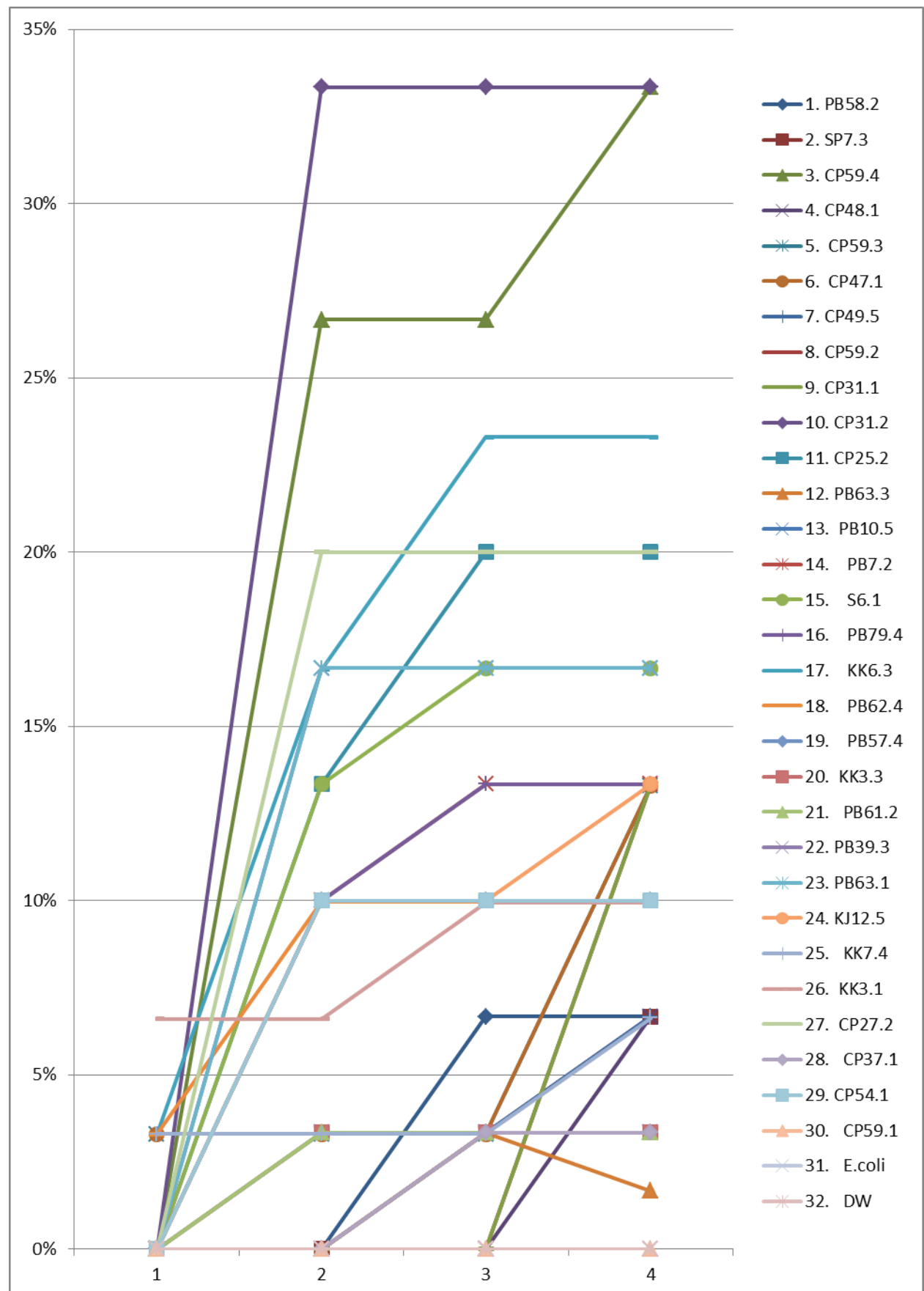


Figure 23 Mortality rate of larva stage 2 of *Aedes* after exposure to bacterial cell pellet of *Xenorhabdus* and *Photorhabdus*

4.2 Insecticide activity of bacterial cell pellet of *Xenorhabdus* and *Photorhabdus* from against larva stage 2 of *Culex*

The mortality rate of larva stage 2 of *Culex* showed 97% on day 3 after exposure to bacterial cell pellet of *Xenorhabdus stockiae* (PB79.4) (Table 22 and Figure 24).

Table 22 Insecticidal activity of bacterial cell pellet of *Xenorhabdus* and *Photorhabdus* against larva stage 2 of *Culex*

Bacteria code	% Mortality rate			
	Day 1	Day 2	Day 3	Day 4
PB58.2	10%	47%	63%	63%
SP7.3	10%	43%	53%	60%
CP59.4	23%	50%	63%	77%
CP48.1	43%	73%	80%	87%
CP59.3	57%	93%	93%	93%
CP47.1	30%	83%	87%	87%
CP49.5	47%	90%	90%	90%
CP59.2	43%	93%	93%	93%
CP31.1	3%	26%	73%	86%
CP31.2	47%	80%	90%	93%
CP25.2	30%	43%	57%	77%
PB63.3	20%	37%	50%	67%
PB10.5	27%	57%	63%	77%
PB7.2	20%	43%	70%	73%
SP6.1	23%	50%	70%	73%
PB79.4	27%	67%	97%	97%
KK6.3	20%	47%	53%	83%
PB62.4	20%	43%	57%	67%
PB57.4	10%	13%	17%	37%
KK3.3	7%	13%	23%	33%
PB61.2	17%	23%	33%	50%
PB39.3	3%	7%	27%	63%
PB63.1	10%	20%	37%	63%

KJ12.5	13%	17%	23%	37%
KK7.4	20%	57%	63%	70%
KK3.1	7%	7%	23%	57%
CP27.2	13%	27%	33%	57%
CP37.1	27%	60%	83%	90%
CP54.1	30%	47%	53%	73%
CP59.1	33%	47%	57%	67%
Control (<i>E.coli</i>)	67%	70%	70%	70%
Control (DW)	80%	90%	90%	90%

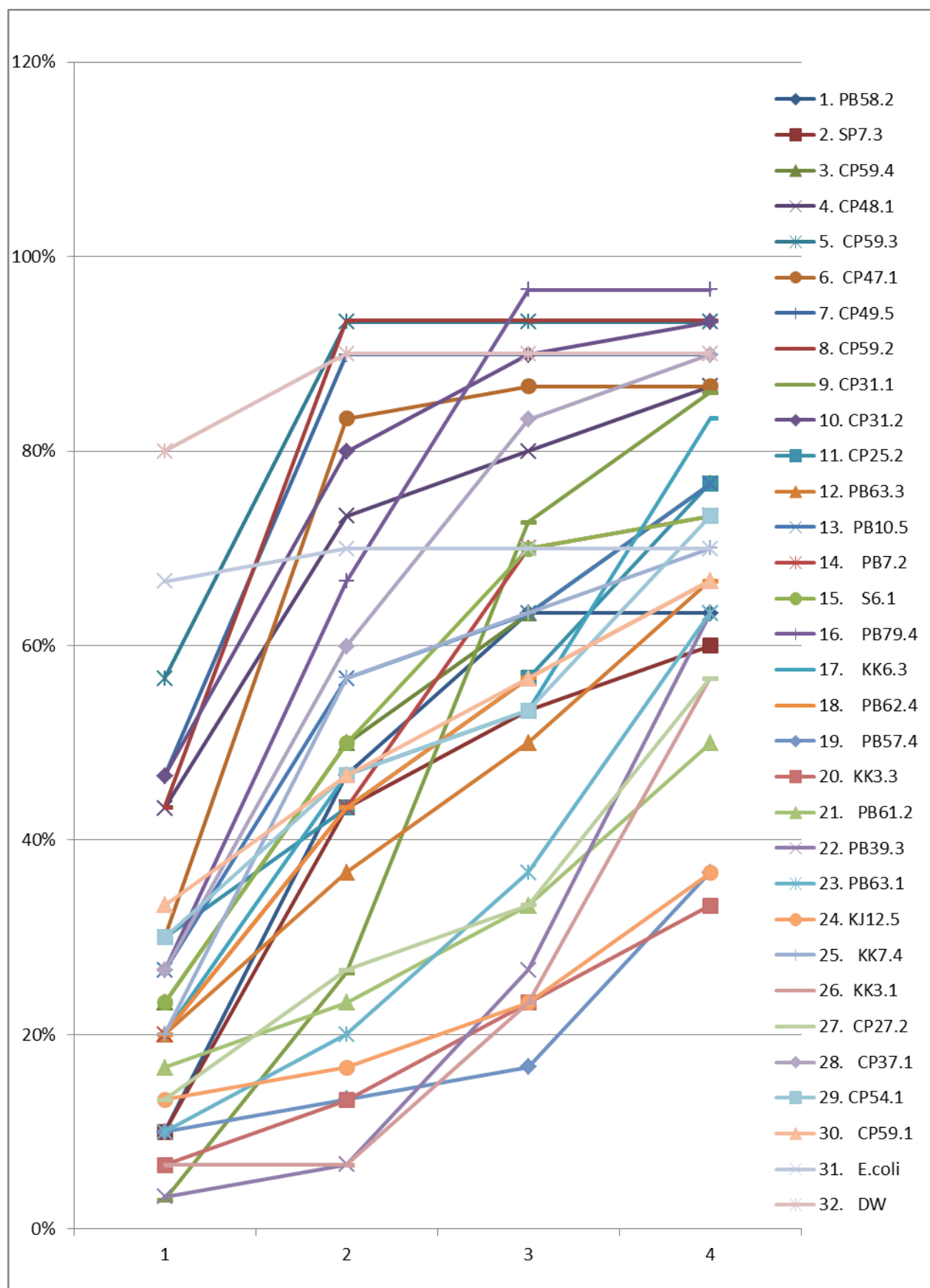


Figure 24 Mortality rate of larva stage 2 of *Culex* after exposure to bacterial cell pellet of *Xenorhabdus* and *Photorhabdus*

4.3 Insecticide activity of bacterial cell pellet of *Xenorhabdus* and *Photorhabdus* from against larva stage 2 of *Anopheles*

The mortality rate of larva stage 2 of *Anopheles* showed 100% on day 2 after exposure to bacterial cell pellet of *Xenorhabdus stockiae* (PB10.5 and PB57.4) (Table 23 and Figure 25).

Table 23 Insecticidal activity of bacterial cell pellet of *Xenorhabdus* and *Photorhabdus* against larva stage 2 of *Anopheles*

Bacteria code	% Mortality rate			
	Day 1	Day 2	Day 3	Day 4
PB58.2	23%	63%	70%	90%
SP7.3	7%	43%	57%	77%
CP59.4	13%	43%	63%	83%
CP48.1	97%	97%	97%	97%
CP59.3	7%	43%	77%	90%
CP47.1	50%	73%	80%	100%
CP49.5	67%	73%	80%	97%
CP59.2	37%	50%	67%	90%
CP31.1	20%	50%	63%	70%
CP31.2	10%	23%	57%	67%
CP25.2	13%	37%	43%	63%
PB63.3	7%	40%	60%	100%
PB10.5	60%	100%	100%	100%
PB7.2	43%	63%	87%	97%
SP6.1	17%	47%	63%	97%
PB79.4	23%	40%	63%	100%
KK6.3	20%	50%	63%	63%
PB62.4	37%	57%	77%	77%
PB57.4	90%	100%	100%	100%
KK3.3	37%	47%	77%	93%
PB61.2	13%	43%	67%	87%
PB39.3	10%	43%	70%	87%
PB63.1	7%	63%	80%	100%
KJ12.5	13%	60%	77%	97%

KK7.4	23%	50%	77%	77%
KK3.1	20%	40%	47%	80%
CP27.2	30%	53%	67%	83%
CP37.1	40%	73%	87%	97%
CP54.1	50%	50%	50%	50%
Control (<i>E.coli</i>)	37%	53%	57%	70%
Control (DW)	0%	0%	0%	0%

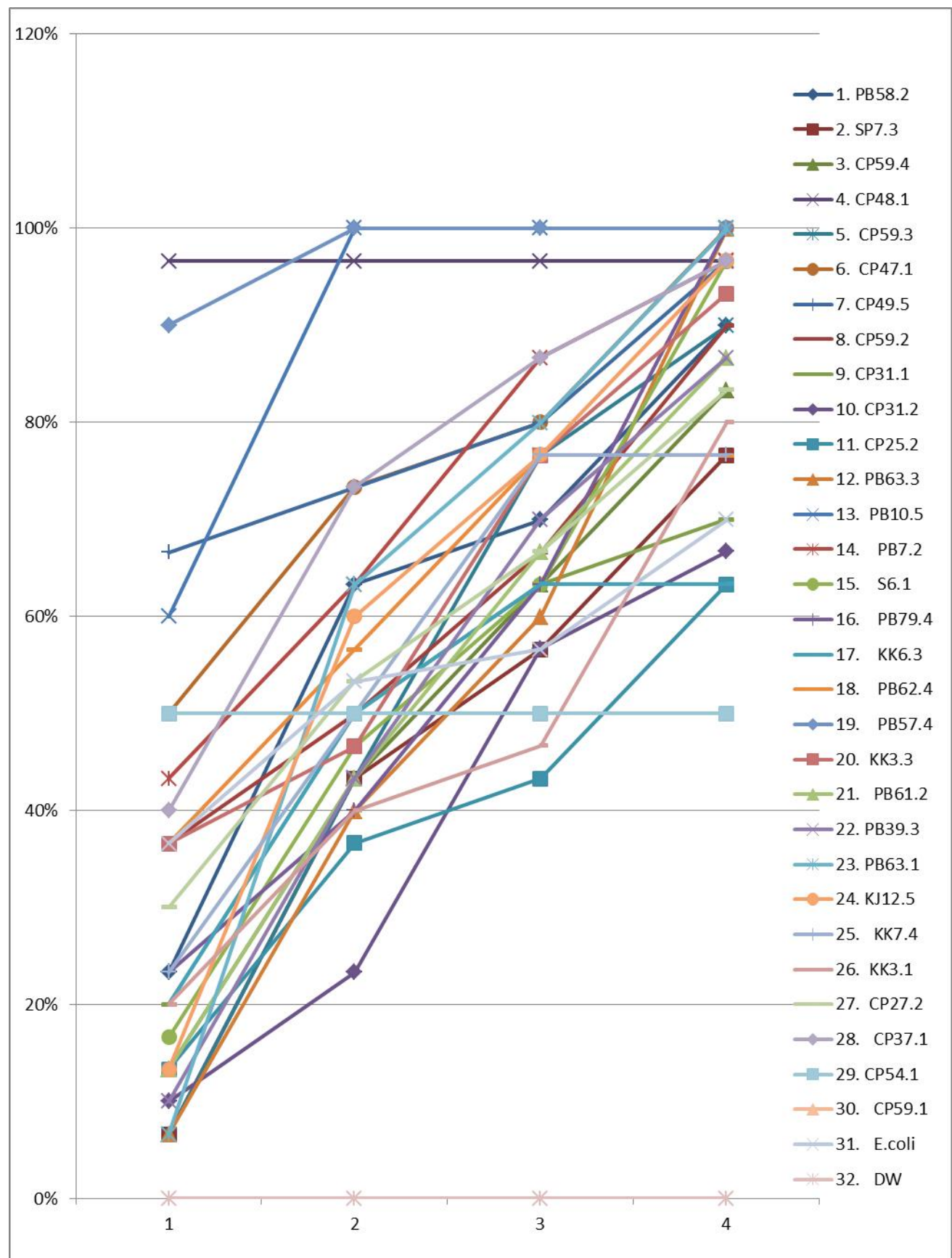


Figure 25 Mortality rate of larva stage 2 of *Anopheles* after exposure to bacterial cell pellet of *Xenorhabdus* and *Photorhabdus*

5 Expression of *PirA/PirB* gene

5.1 Polymerase Chain Reaction of *PirA/PirB* gene

Sequence length of PirA and PirB of *Photorhabdus luminescence* was approximately 400 and 1,300 bp, respectively (Figure 26 and 27).

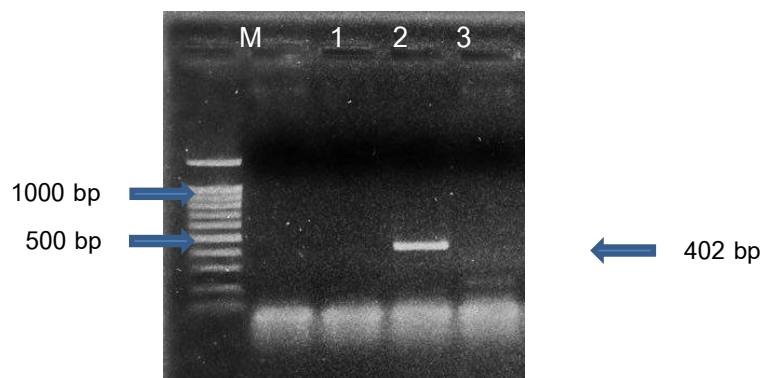


Figure 26 PCR product of PirA (Lane 3) and negative control (Lane 4)

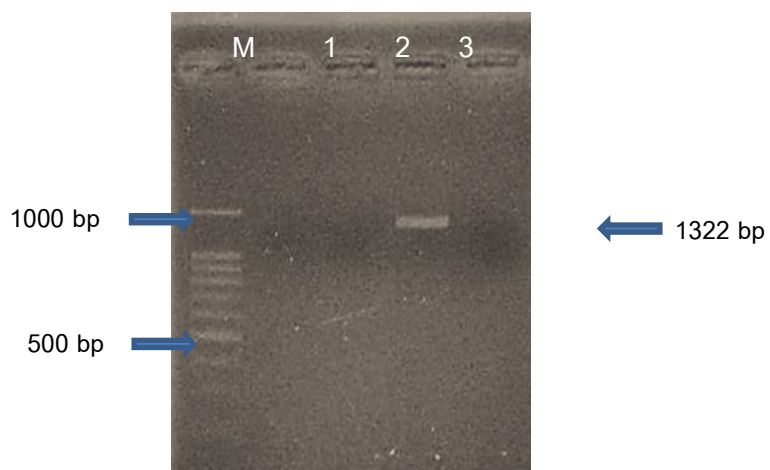


Figure 27 PCR product of PirB (Lane 3) and negative control (Lane 4)

5.2 Cloning

pirA and PirB gene were clone into pET32a (expression vector) and then transformed to BL21 (*E. coli*) (Figure 28 and 29). Colony PCR was performed for checking insert gene.

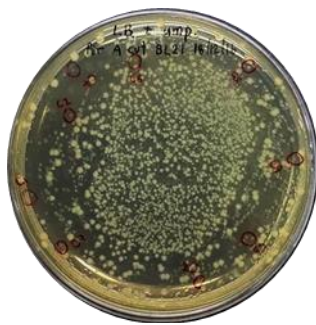


Figure 28 PirA transformant colonies on LB agar containing ampicillin (100 µg/ml)

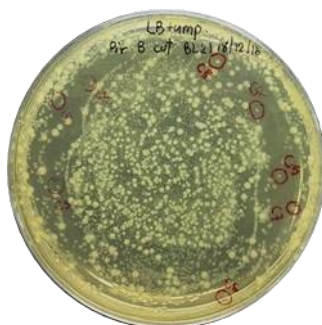


Figure 29 PirB transformant colonies on LB agar containing ampicillin (100 µg/ml)

5.3 Protein expression of *PirA* and *PirB*

Protein expression of *PirA* and *PirB* were not clear both clones that induced with IPTG and uninduced with IPTG. Therefore, western blot was performed to confirm. *PirA* and *PirB* expression should be 14.8 kDa and 46.2 kDa, respectively. SDS PAGE showed *PirA* (Figure 30) and *PirB* (Figure 31) after induced with IPTG (100 mM) at 37°C for 4 h, respectively.

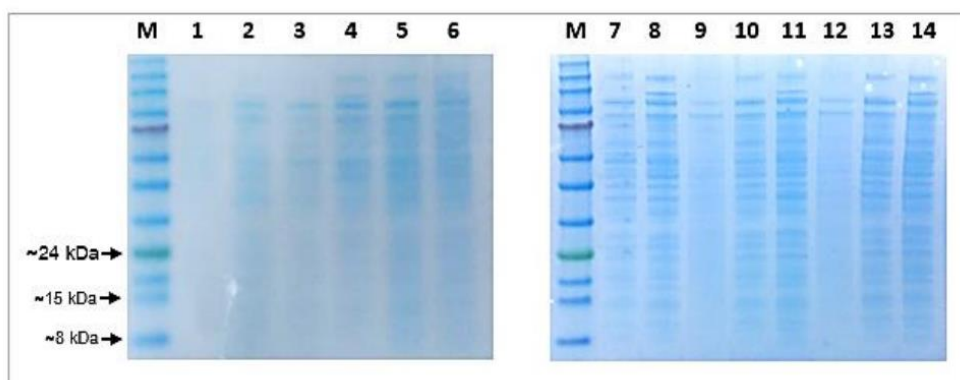


Figure 30 SDS PAGE of protein expression of *PirA* after induced with IPTG (100 mM) and uninduced. Lane M Protein marker, Lane 1, 3, 5, 7, 9, 11, and 13 were uninduced with IPTG of clone 1-7, respectively; Lane 2, 4, 6, 8, 10, 12, and 14 were induced with IPTG of clone 1-7, respectively

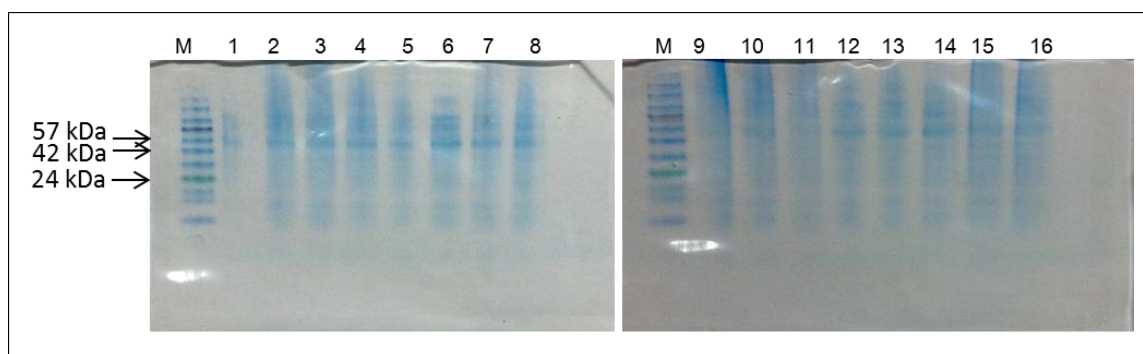


Figure 31 SDS PAGE of protein expression of PirB after induced with IPTG (100 mM) and uninduced. Lane M Protein marker, Lane 1, 3, 5, 7, 9, 11, 13, and 15 were uninduced with IPTG of clone 1-8, respectively; Lane 2, 4, 6, 8, 10, 12, 14 and 16 were induced with IPTG of clone 1-8, respectively;

5.4 Western Blot

Western blot of PirA and PirB were performed by using His Detector Nickel conjugate bind to His tag on recombinant clone. Protein expression of PirA and PirB were not difference between clones that induced with IPTG (100 mM) and uninduced (Figure 32 and 33).

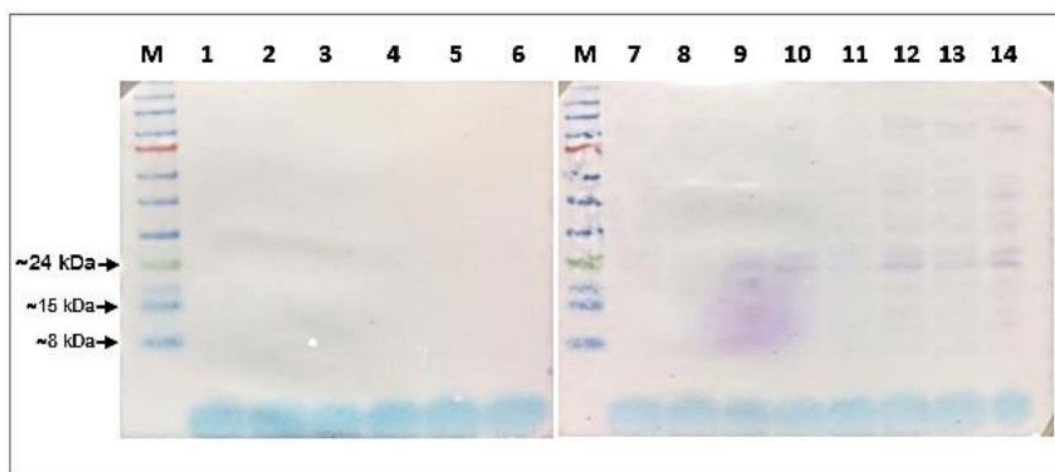


Figure 32 Western Blot of PirA on Nitrocellulose Membrane after using His Detector Nickel conjugate bind to His tag on recombinant clone. Lane M Protein marker, Lane 1, 3, 5, 7, 9, 11, and 13 were uninduced with IPTG of clone 1-7, respectively; Lane 2, 4, 6, 8, 10, 12, and 14 were induced with IPTG of clone 1-7, respectively;

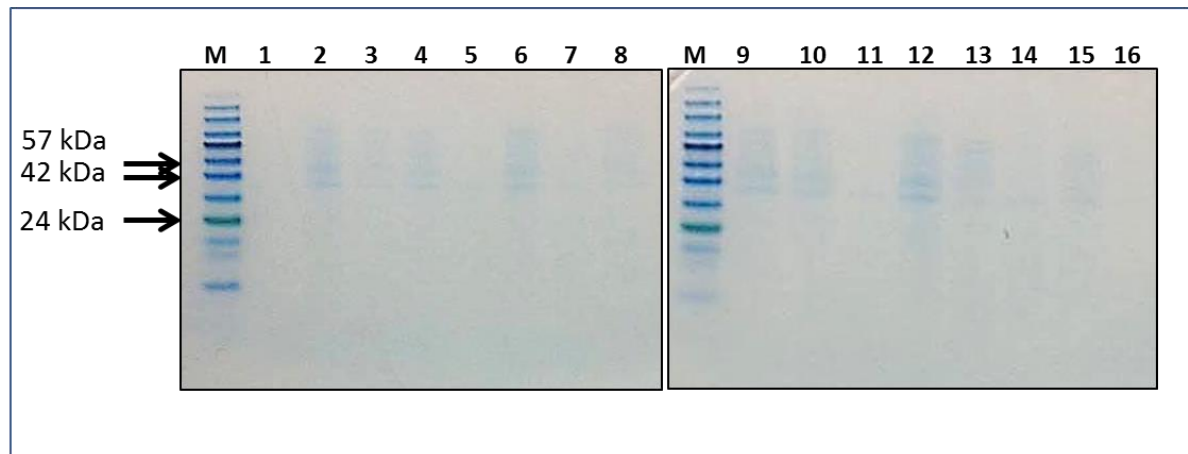


Figure 33 Western Blot of PirB on Nitrocellulose Membrane after using His Detector
Nickel conjugate bind to His tag on recombinant clone. Lane M Protein marker, Lane 1, 3, 5, 7, 9, 11, 13, and 14 were uninduced with IPTG of clone 1-8, respectively; Lane 2, 4, 6, 8, 10, 12, 14, and 16 were induced with IPTG of clone 1-8, respectively;

Conclusion and Discussion

Insecticide activity of whole cell suspension, Cell-free supernatant, Crude cell extract and Bacteria cell pellet of *Xenorhabdus* and *Photorhabdus* against larva stage 1-2 and 3-4 of *Aedes*, *Culex* and *Anopheles*

Whole cell suspension of *Photorhabdus luminescens* subsp. *hainaensis* (KK1.3) and *Xenorhabdus miraniensis* (PB62.2) showed high effective to kill larva stage 1-2 of *Aedes* and *Culex*. Furthermore, Whole cell suspension of *Photorhabdus luminescens* subsp. *hainaensis* (KK1.3) and *Photorhabdus luminescens* subsp. *akhurstii* (NK2.5) showed effective to kill larva stage 3-4 of *Culex*. Cell free supernatant of *Photorhabdus luminescens* subsp. *hainaensis* (KK1.3) showed high effective to kill larva stage 1-2 of *Aedes*. In addition, cell free supernatant of *Xenorhabdus miraniensis* (PB62.2) and *Xenorhabdus stockiae* (PB57.4) showed high effective to kill larva stage 1-2 of *Aedes* and larva stage 3-4 of *Culex*. Crude cell extract of *Xenorhabdus stockiae* (PB57.4) and *Photorhabdus luminescens* subsp. *akhurstii* (NK2.5) can kill larva stage 3-4 of *Culex*, whereas, bacteria cell pellet of *Photorhabdus luminescens* subsp. *hainaensis* (KK1.3) and *Photorhabdus luminescens* subsp. *akhurstii* (NK2.5) can also kill larva stage 3-4 of *Culex*. Furthermore, bacteria cell pellet of 2 isolates of *Xenorhabdus stockiae* (PB10.5 and PB57.4) can kill larva stage 2 of *Anopheles*. However, protein (toxin) from recombinant PirA and PirB gene cannot express. It may be toxic to bacterial cell.

From this result, these isolates may be due to can produce bioactive compounds that are effective in killing mosquito larva. Previously study, *Xenorhabdus nematophila* can produce compounds with insecticidal properties include toxin complexes (Tc's) or lipopolysaccharides (Owuama, 2001; French-constant, et al., 2007; Hinchliffe, et al., 2010). This indicates that *Xenorhabdus* and *Photorhabdus* may be an alternative biological agents for *Aedes*, *Culex* and *Anopheles*. However, the isolation of compounds produced by *Xenorhabdus* and *Photorhabdus* are needed to investigate for more understanding.

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Appendix

6. Output (Acknowledge the Thailand Research Fund)

8.1 International Journal Publication

Manuscript in title Oral toxicity of *Xenorhabdus* and *Photorhabdus*
from Thailand against mosquito larvae (In preparation)

8.2 Research Utilization and Application

8.3 Others e.g. national journal publication, proceeding, international conference, book chapter, patent