Emir. J. Food Agric. 2011. 23 (01): 17-26
http://ejfa.info

Quick isolation and characterization of novel *Bacillus thuringiensis* strains from mosquito breeding sites in Malaysia

Kavitha Renganathan[1], Xavier Rathinam[1*], Monica Danial[2] and Sreeramanan Subramaniam[2]

[1]AIMST University, Bukit Air NAsi, Jalan Bedong, 08100, Kedah, Malaysia; [2]School of Biological Sciences, Universiti Sains Malaysia, Gelugor, 11800 Penang, Malaysia

Abstract: *Bacillus thuringiensis* (*Bt*.) is an insecticidal, gram-positive and spore forming bacterium which has capabilities infecting the insect vectors belonging to the order Diptera (mosquitoes) especially dengue causing *Aedes* species. Bacterial strains with combination of Cry and Cyt proteins are useful in controlling and management of resistance in mosquito population. The objective of this research is to isolate and characterize *Bacillus thuringiensis* from mosquito breeding sites and also study expression of Crystal (Cry) and Cytolytic (Cyt) proteins of these isolates by SDS-PAGE analysis. In this study, 50 samples were collected from mosquito breeding sites which include soil, larvae and water. Samples were processed by initially giving heat shock treatment, serial dilution and followed by plating onto culture medium. Initial selection was based on colony morphology, gram staining, phase contrast microscopy and coomassie blue staining. Total of 144 isolates was identified based on colony morphology and this number was reduced to 69 after gram staining. Total of 15 isolates were confirmed to be *Bacillus* after phase contrast microscopy. Through coomassie blue staining, a blue cap-like appearance in the sporulation phase and darkly stained oval shaped candidate in autolysis phase can be observed. Growth curve analysis was done with one chosen isolate to determine the generation time. Further protein level characterization based on the SDS-PAGE gel showed the banding patterns range from 65-274 kDa (Cry proteins) and 21-25 kDa (Cyt proteins).

Keywords: *Bacillus thuringiensis*, Mosquito breeding site, Biochemical analysis, Growth curve analysis, SDS-PAGE

العزل السريع وتشخيص سلالات جديدة من بكتيريا *Bacillus thuringiensis* من مواقع تربية البعوض في ماليزيا

كبفيتا رينقاناتن [1] ، سيفيرت رتنم [1*] ، مونيكا دانيال [2] و سريرامانان سوبرامانييم[2]

[1] جامعة AIMST، بوكت اير ناسي ، جلن بيدونق ، 08100 ، كيدا ، ماليزيا ;[2] مدرسة علوم البيولوجيا ، جامعة سين – ماليزيا ، جلوجر ، 11800 بيناناق ، ماليزيا

الملخص:تعد بكتيريا *Bacillus thuringiensis* مبيد حشري، موجب الصبغ بصبغة جرام و يكون جراثيم بكتيرية لها القدرة على اصابة العوائل من رتية ثنائية الأجنحة (Diptera, mosquitoes) وخاصة النوع Aedes المسبب لمرض dengue . السلالات البكتيرية الحاملة لتشكيلات من بروتينات Cry و Cyt تكون مفيدة في تحجيم وادارة المقاومة لعشائر البعوض. كان الهدف من هذا البحث هو عزل وتشخيص *Bacillus thuringiensis* من مواقع لتربية البعوض، وأيضاً دراسة تعبير بروتينات كريستال (Cry) والتحليل الخلوي (Cyt) من هذه العزلات باستخدام تقنية SDS-PAGE. في هذه الدراسة جمعت 50 عينة تمثل التربة، الشرنقة والمياه. تم معاملة العينات بتعرضهم لصدمة حرارية مبدئية، التخفيف المتتالي الذي تبعه الزراعة علي البيئات الصناعية. تأسس الإنتخاب الأولي عليالشكل الظاهري للمستعمرة، الصبغ بصبغة جرام، الفحص الميكروسكوبي والصبغ بصبغة coomassie blue. تم تعريف 144 عزلة بناءً على الشكل الظاهري وتم إنقاصه إلى 69 بعد الصبغ بصبغة جرام. تم التأكد من 15 عزلة من *Bacillus* بعد الراسة الميكروسكوبية. باستخدام صبغة coomassie blue ظهرت منطقة شبيهة لمنطقة القمة الزرقاء في مرحلة التجرثم ومنطقة عامقة اللون بيضاوية الشكل في مرحلة التحلل الذاتي. تم وضع منحنى النمو باستخدام عزلة واحدة لتقرير طول فترة الجيل. كما أظهر التشخيص باستخدام تقنية SDS-PAGE شرائط تتراوح من 65-274 kDa لبروتيات (Cry) و 21-25 kDa لبروتينات (Cyt).

* Corresponding Author, *Email*: rxavier@yahoo.com

Introduction

B. thuringiensis is a gram-positive, rod shaped and facultative anaerobe bacterium present in soil, water and on plant surface (Gill et al., 1992; Moraga et al., 2004). They have high degrees of insect specificity and environment safe, thus making it suitable for use against mosquito in wetlands and those resistant to synthetic chemical insecticides (Hofte et al., 1989; Schnepf et al., 1998; Xavier et al., 2007).

Bacterial toxicity is due to endotoxin proteins, which are synthesized during sporulation and assembled into parasporal crystals that are toxic when ingested by larvae (Kronstad et al., 1983; Crickmore et al., 1998; Schnepf et al., 1998). Endotoxins produced by *Bacillus* species are often species specific and they do not contaminate the environment as the toxins have lesser residual efficacy and are generally safe for non-target organisms (Lacey and Undeen, 1986; Siegel, 2001; Moraga et al., 2004; Crickmore, 2006). This is due to the presence of insecticidal crystal proteins (ICP) which are biodegradable and environmental friendly (Ibarra et al., 2003). ICP are produced by different subspecies that exhibits specific insecticidal activities towards the larvae of members Lepidoptera, Diptera and Coleoptera. Upon ingestion, the ICPs are solubilized and hydrolyzed by high pH (9 to 11) and specific proteases of in insects' midgut (Dean, 1998). The hydrolytic products bind to receptors on the midgut epithelium and forms pores. It then results in osmotic lysis of epithelial cells and eventually fatal (Feitelson et al., 1992; Crickmore et al., 1998; Schnepf et al., 1998). ICPs of *B. thuringiensis* subsp. *israelensis* are specifically lethal to some members of the order Diptera (Thomas and Ellar, 1983). The hydrolysis of parasporal body in the larval gut results in four major Cry proteins known as with molecular mass of 65kDa, 128kDa and 135kDa and 270kDa (Van Rie et al., 1992).

Biopesticides are created as chemical methods of controlling pests are detrimental to human as well as the environment. Since mosquitoes are able to spread diseases such as dengue and malaria, *B. thuringiensis* based biopesticides are developed to control these mosquitoes (Alan et al., 1993; Georghion and Wirth, 1997; Oliveira et al., 2003). The specific activity of *B. thuringiensis* is considered as highly beneficial. Unlike most insecticides, *B. thuringiensis* does not kill beneficial insects as it would be specific. This includes natural enemies such as parasites and pollinators like honey bees Federici, 2005). Thus, *B. thuringiensis* integrates well with other biological controls (Crickmore, 2006). Since *B. thuringiensis* is host specific, it is non toxic to humans and animals if they happened to be exposed to it (Siegel, 2001). This high margin of safety recommends its use on food crops or in other sensitive sites where pesticide use can cause adverse effects (Cranshaw, 2006). Due to intensive research on pesticidal activity of *B. thuringiensis*, information regarding the structure, mode of action and crystal proteins regulation emerged. In order to control mosquito larvae, formulations containing *israelensis* strain is placed into the standing water of mosquito breeding sites (Georghion and Wirth, 1997). For these applications, *B. thuringiensis* is formulated as solid or granules, slow release rings or brickettes to increase persistence. The size of water body determines the rate of use. The application is carried out shortly after insect eggs are expected to hatch which happens after flooding due to rain or irrigation. Although the *israelensis* strain is quite specific in its activity, some types of non-biting midges, which serve as food for fish and wildlife, also are susceptible and may be affected (Georghion and Wirth, 1997). There are several insects controlled by *B. thuringiensis* of different strains. *Kurstaki* strain controls vegetable insects, field and forage crop insects, fruit crop insects, tree

and shrub insects. *Israelensis* strains controls mosquitoes, black flies and fungus gnat. *San diego*/*tenebrionis* strains on the other hand controls Colorado potato beetle, elm leaf beetle and cottonwood leaf beetle (Cranshaw, 2006).

The objectives of this research is to isolate and characterize *Bacillus thuringiensis* from mosquito breeding sites and also study expression of Crystal (Cry) and Cytolytic (Cyt) proteins of these isolates by SDS-PAGE analysis.

Materials and Methods

Sample collection

Samples which include mosquito larvae, water and soil were collected from different mosquito breeding sites. The total volume of sample collected was 30 ml and soil layer with a depth of 1 cm was removed. The collected samples was placed into a 50 ml plastic container and sealed. Each sample was then labelled appropriately. Total of 50 samples were collected using this method (Xavier et al., 2007).

Sample processing

The collected samples in the plastic container were vigorously shaken to allow an even mixture between the water and soil. 10 ml of sample was measure and placed into sterile 250 ml conical flasks containing 90 ml of sterile distilled water. The conical flasks were then covered with cotton plug and placed on an orbital shaker set at 250 rpm for 4 hours. This procedure ensures the microbe attached to the soil particle is dispensed into the distilled water and to ensure the samples are homogenized. After 4 hours, 1 ml of the soil-water samples were collected using a sterile pipette and immediately placed into 6 ml boiling tubes. These boiling tubes were then subjected to heat shock treatment in a water bath set at 80°C for 10 minutes. This step is to ensure all microbes in vegetative form are killed, leaving behind only the microbial spores. After heat shock treatment, the samples were vortexed and three-fold serial dilutions were done. Serial dilution was done with addition of 100 µl of sample into 900 µl sterile distilled water. The serially diluted sample suspensions ranging from 10^{-1} to 10^{-3} were plated onto Nutrient Agar plate using spread plate technique. These plates were then incubated for 24 to 48 hours at 30°C in an incubator. The potential *Bacillus* colonies which grew on the plates were further characterized using morphological and biochemical characterization.

Colony morphology identification

The colonies were found to be white to off-white in colour with smooth edges and flat to slightly raised elevation (Rampersad and Ammons, 2005) were selected and marked on the Petri dish. The selected colonies with those characteristics were categorized as possible *Bacillus* colonies. Total of 144 colonies were chosen based on colony morphology. These selected colonies were then sub-cultured onto new Nutrient Agar plates and incubated.

Gram staining

Gram staining technique is used to confirm the bacterial colonies to be gram positive. The sample is smeared onto a slide with a drop of distilled water, followed by air drying and heat fixing. Then, gram staining procedure was carried out with primary staining with crystal violet, followed by fixation with iodine, decolouration with acetone and lastly counter staining with safranin. The slides was then left to air dry and observed under light microscope. This procedure was repeated for all 144 colonies. Blue coloured cells are gram positive where as pink coloured cells are gram negative. Total of 69 colonies had rod shape morphology and were gram positive.

Phase contrast microscopy

The gram positive colonies were then inoculated into sterilized 250 ml conical flasks containing 50 ml Nutrient Broth and placed on an orbital shaker set at 250 rpm for 4 days. The purpose is to induce sporulation of the bacterial cells. Wet

mount slides were prepared after 4 days from the inoculated Nutrient broth. These slides were observed under phase contrast microscope (Olympus BX50 fitted with a JVC K-F55B colour video camera and analysis Docu Version 3.1 image analysis system) in order to visualize the endospore and parasporal bodies which are important in *Bacillus thuringiensis* identification. Besides that, wet mount was also done after 2 days of inoculation in order to visualize the vegetative phase. Only 15 isolates possessed criteria under *Bacillus* which is having both endospores and parasporal bodies. However, these isolates are further characterized through Coomassie Blue Staining for better confirmation.

Coomassie Blue Staining (CBB)

Coomassie blue staining was done for two different phases in *Bacillus* life cycle which are sporulated phase and autolysis phase. This staining method increase resolution where by the sporulated phase can be easily viewed. The 15 *Bacillus* isolates were inoculated individually into sterilized 250 ml conical flasks containing 50 ml Nutrient broth. The conical flasks were left on the orbital shaker set at 250 rpm for 90 hours (sporulation phase) and 110 hours (autolysis phase). Samples were smeared onto glass slides, followed by air dry and heat fixing. Smear slides were prepared for both phases of the *Bacillus* life cycle. These slides was stained using 0.133% coomassie blue staining solution and destained using destaining solution. Observation under microscope was done to view blue colour stained parasporal bodies in the *Bacillus* cells during sporulation phase and the ICP during autolysis phase. All 15 potential *Bacillus* isolates showed stained parasporal bodies and ICP which are confirmed to be *Bacillus thuringiensis*. These isolates were then subjected to biochemical characterization using SDS-PAGE analysis to confirm the isolates being *Bt*.

Biochemical characterization

SDS-PAGE analysis was carried out. All 15 potential *Bacillus thuringiensis* strains were inoculated individually in 50 ml Nutrient broth for 4 days in order to reach complete autolysis phase.

Protein extraction

After complete autolysis, 10 ml of the Nutrient broth containing lysed cells were pipetted into a 50 ml Beckman's centrifuge tube. Crude protein was isolated by centrifugation at 10,000 rpm at 4°C for 15 minutes. The supernatant was discarded and the pellet was resuspended in 2 ml of sterilized distilled water.

Protein purity

Protein purity was measured using UV spectrophotometer in order to determine the purity. Absorbance reading of crude protein was taken at 260nm and 280nm. The ratio of absorbance at 280nm over 260nm was calculated. Pure protein would have values ranging from 1.8 to 2.0. The crude protein had a value 1.72 which is almost close to 1.8. Protein quantification was carried using Bradford Method (1976).

Growth curve analysis

Using one of the *Bt*. strains, a growth curve was generated. The chosen *Bt*. strain was inoculated in sterilized 250 ml conical flask containing 50 ml Nutrient broth. The growth was generated by taking the absorbance readings at 595 nm at 5 hours interval beginning at 0 hour till 120 hours.

SDS-PAGE analysis

SDS gel electrophoresis was performed on the protein samples according to a method by (Lamelli, 1970).

Results

Colony morphology

A typical *Bacillus* colony was identified based on the characteristics mentioned earlier (Figure 1). A total of 144 colonies were isolated and sub cultured in fresh Nutrient Agar plates to obtain single colonies.

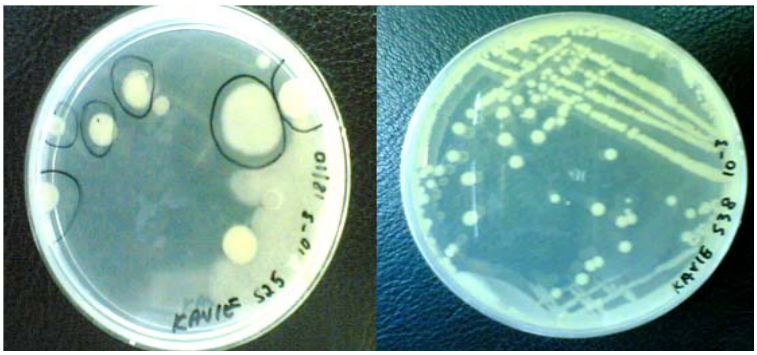

Figure 1. Creamy white colonies indicating a *Bacillus* colony (left). Single colonies obtained after sub-culturing (right).

Gram staining

Bacillus species are generally gram positive and rod-shaped (Figure 2). Gram staining was done to differentiate gram positive from the gram negative. Light microscope was used for observations. Isolates which were rod shaped and blue in colour indicates a gram positive strain where as isolates which did not exhibit these characteristics were discarded. Under the microscope, the vegetative cells of *Bacillus* are thin and long. In contrast, sporulated cells were thick and short.

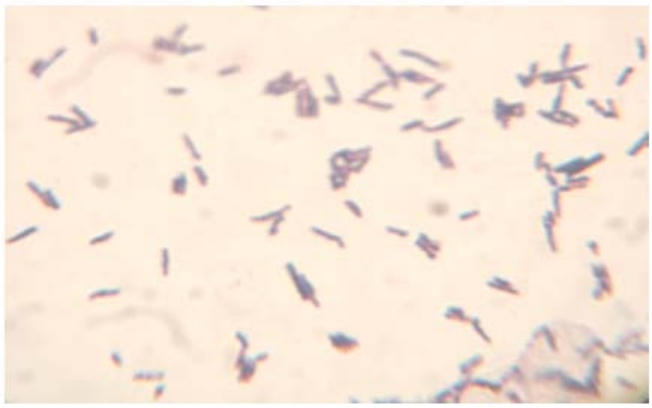

Figure 2. Vegetative form of *Bacillus* (rod shaped and thin) at 1000X magnification. Gram positive (blue).

Phase contrast microscopy

Phase contrast is carried out after determining the colonies are gram positive through gram staining. This procedure is important to confirm the isolates are *Bacillus* by viewing the endospore and parasporal bodies. Besides that, phase contrast microscopy was also done for vegetative phase cells to confirm the isolates were rod shape (Figure 3). Wet mount slides were prepared after 4 days of incubation for each isolate. Isolates that showed presence of both endospores and parasporal bodies were selected for further characterization and isolates without those characteristics were discarded (Figure 4). A total of 15 isolates possessed presence of endospores and parasporal bodies.

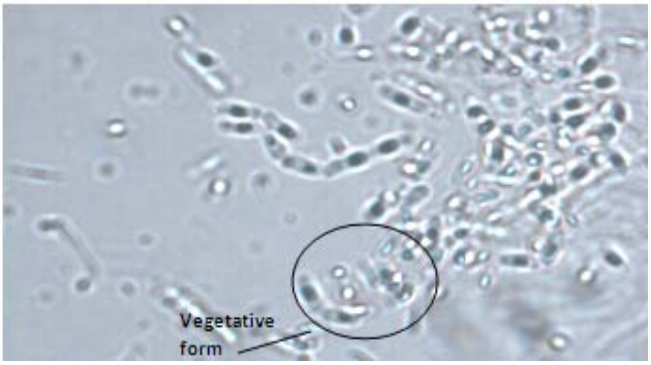

Figure 3. Vegetative form of *Bacillus* at 1000X magnification with endospore and parasporal body attached together.

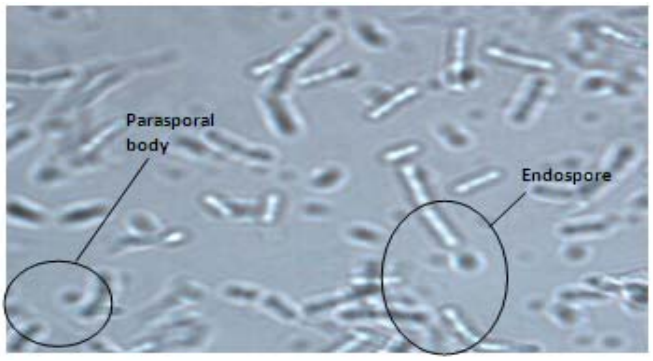

Figure 4. Sporulated form of *Bacillus* at 1000X magnification.
Parasporal body is an dark oval region and the endospores are bright rod shape.

Coomassie Blue Staining (CBB)

Total of 15 isolates were then subjected to further screening of parasporal body through coomassie blue staining (Figure 5). This method has a higher resolution compared to phase contrast microscopy. Thus, samples which have parasporal body can be easily identified. All 15 isolates took up the coomassie blue stain and had parasporal bodies during the sporulated phase and autolysis phase (Figure 6).

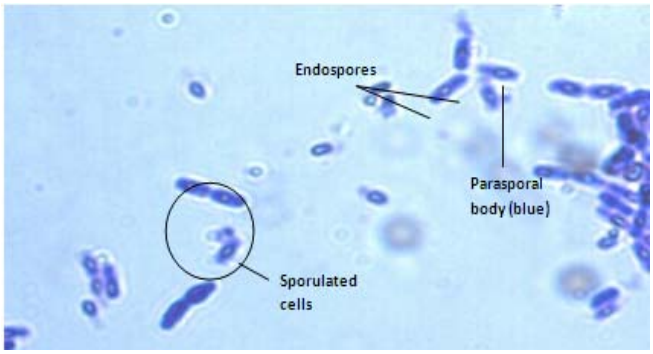

Figure 5. Sporulation phase of *Bt.* isolates with blue stained parasporal body at 1000X magnification.

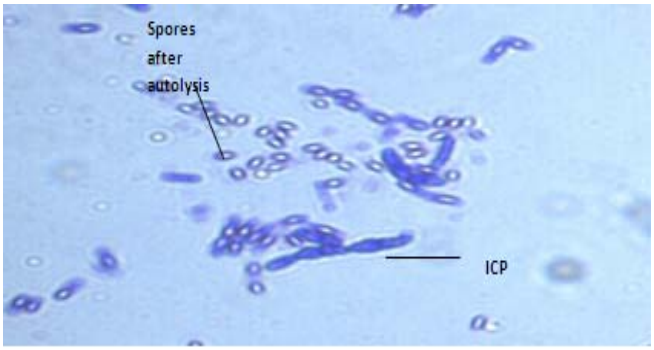

Figure 6. Cell lysis phase of *Bt.* isolates with blue stained ICPs and bright coloured spores with blue lining at 1000X magnification.

Growth curve analysis

One strain was used to generate a growth curve and it was plotted based on absorbance reading 595 nm at an interval of 5 hours for 120 hours (Figure 7). Based on the growth curve, the isolated strain sporulated at 90 hours after inoculation in Nutrient broth at 250 rpm. This shows a similar sporulation time as a standard *Bt.* strains which is approximately 92 hours (Bulla et al., 1980).

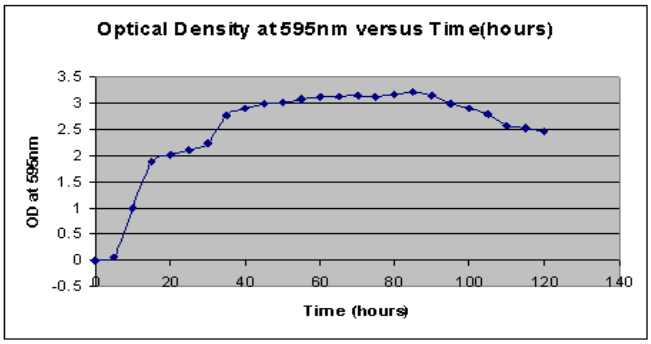

Figure 7. Growth curve of a Bt. isolate which sporulated at 90 hours.

SDS-PAGE analysis

SDS-PAGE analysis was carried out for all 15 isolates. However, siblings were eliminated leaving only one sibling for SDS-PAGE. Total of 9 isolates were selected based upon the prominent Cry and Cyt proteins. Based on the SDS-PAGE gel, the banding patterns range from 65- 274 kDa (Cry proteins) and 21-25 kDa (Cyt proteins).

Discussion

Due to the development of resistance in mosquitoes to chemical pesticides much of the research is being directed to explore the efficacy of biocontrol agents such as pathogenic bacteria and other organisms. Many of these organisms have been isolated from insects under natural habitat conditions. However only few of Bt strains could be considered as candidates for biocontrol (Crickmore et al., 1998; Schnepf et al., 1998, Deacon, 2001; Siegel et al., 2001, Zhang et al., 2000). Mosquitoes are vectors of many human infectious diseases. The most important disease vectors are members of subfamilies Anopheliae (*Anopheles* mosquitoes) which transmits malaria; Culicidae (*Culex*

mosquitoes), *Aedes* mosquitoes which transmits filariasis and dengue respectively (Service, 1986). The increasing problems pertaining mosquitoes and the use of *Bt*. products as biopesticide inspired the work to pursue this research of isolation and characterization of *Bacillus thuringiensis* from mosquito breeding sites. This study presents information on the occurrence of spore forming bacilli bacteria in the bodies of mosquito larvae as well as in the soil of their natural breeding sites (Mohsen et al., 1986).

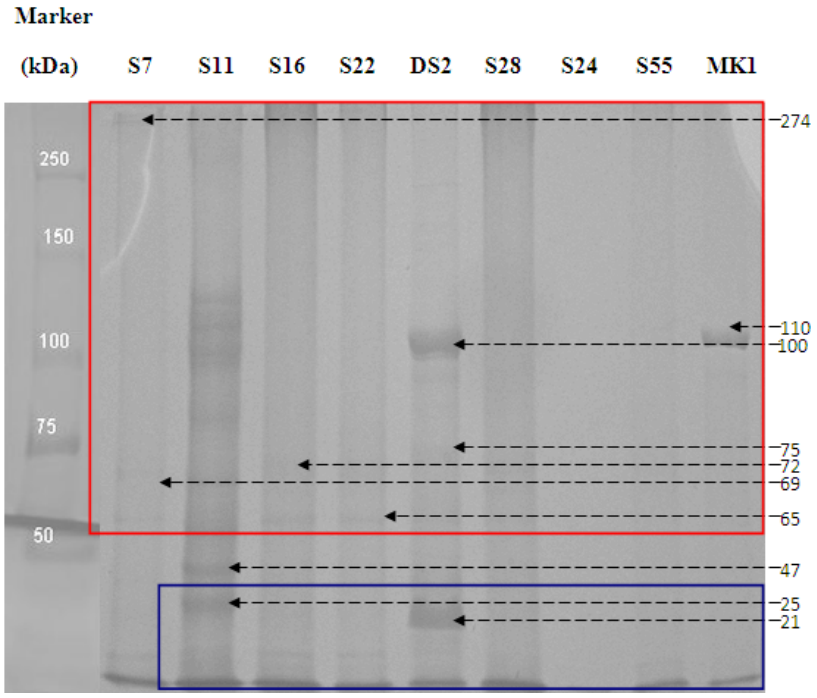

Figure 8. Proteins ranging from 65-274 kDa (red box) are Cry proteins. Cyt proteins ranges from 21-25 kDa (blue box).

The results show that *Bacillus thuringiensis* strains are found in mosquito breeding sites. In this research, *Bacillus* was isolated by using heat shock to eliminate vegetative cells leaving behind the endospores. A total of 149 colonies were isolated based on the colony morphology showing white to off-white colour with smooth edges and flat to slightly raised elevation (Rampersad and Ammons, 2005). Gram staining was carried out to determine gram positive bacteria. This procedure confirmed 69 isolated samples were gram positive *Bacillus*. All 69 samples was then inoculated in Nutrient broth and left for agitation at 250 rpm. These samples were then taken at 50 hours and 90 hours of incubation to view vegetative phase and sporulated phase respectively under phase contrast microscopy. Endospores and parasporal body could be observed during sporulation phase. Thus, 15 out of 69 samples possessed those characteristics. All 15 samples were subjected to CBB staining to confirm the presence of parasporal body during sporulation phase and autolysis phase. Autolysis phase bacterial cells were taken after 110 hours of inoculation in Nutrient broth at 250 rpm. All 15 sporulated samples showed dark blue coloured parasporal body where as autolysed samples showed blue coloured crystal proteins and unstained endospore under phase contrast microscope. Based on these observations, all 15 samples were

confirmed to be *Bacillus thuringiensis*. Therefore, further biochemical characterization was done through SDS-PAGE analysis (Lamelli, 1970).

One strain was used to generate a growth curve based on absorbance at 595 nm at 5 hours intervals. Readings were taken beginning from 0 hour until 120 hours. From the generated growth curve, the isolated strain sporulated at 90 hours in Nutrient broth. This sporulation time is almost similar to Bulla et al., 1980 which is approximately 92 hours. In order to carry out SDS-PAGE, crude protein was extracted after autolysis and protein quantification done with Bradford method. Through many trials of SDS-PAGE, isolates that had siblings and those that did not show clear Cry and Cyt proteins were discarded. Finally, nine isolates were used in final SDS-PAGE. However, 3 strains (S24, S28, and S55) did not show any banding patterns. This could be due to protein degradation before SDS-PAGE was carried out. 4 strains (S7, S16, S22 and MK 1) showed presence of Cry proteins, 1 strain (S11) with presence of Cyt protein and 1 strain (DS2) with both Cry protein and Cry protein. Since S11 and DS2 have Cyt proteins, assumption can be done that both might have mosquitocidal properties. Further molecular characterization through PCR can be carried out to confirm the strain. Since *Bt*. is host specific, the use of *Bt*. based biopesticides would be safe to humans and the environment. Such new a isolate can be best utilized in combating the vectors in an effective manner. Further the commercialization of this isolate may play a major role in the integrated vector control programs and to manage the insect resistance.

Acknowledgements

This work was supported by AIMST University and Universiti Sains Malaysia.

References

Alan G. Porter, Elizabeth W. Davidson and Jian-Wei Liu. 1993. Mosquitocidal toxins of Bacilli and their genetic manipulation for effective biological control of mosquitoes. Microbiol. Rev. 57(3):838-861.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Bulla, L. A, D. B. Bechtel, K. J. Kramer, Y. I. Shethna, A. I. Aronson and P. C. Fitz-James. 1980. Ultra structure, physiology and biochemistry of *Bacillus thuringiensis*. Crit. Rev. Microbiol. 8:147-204.

Cranshaw, W. S. 2006. *Bacillus thuringiensis*. CSU Cooperative Extension 5556.

Crickmore, N. 2006. Beyond the spore-past and future developments of *Bacillus thuringiensis* as a biopesticide. J. App. Microbiol. 101:616-619.

Crickmore, N., D. R. Zeigler, J. Feitelson, E. Schnepf, J. Van Rie, D. Lerecius, J. Baum and D. I. Dean. 1998. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62:807-813.

Deacon, J. 2001. The Microbial World: *Bacillus thuringiensis*. Institute of Cell and Molecular Biology. University of Edinburgh.

Dean, D. H. 1995. Revision of the nomenclature for *Bacillus thuringiensis* pesticide *cry* genes. In: Program and Abstracta of the 28th Annual Meeting of the Society for Inverteb. Pathol. 14.

Federici, B. A. 2005. Insecticidal bacteria: An over whelming success for invertebrate pathology. J. Invert. Pathol. 89:30-38.

Feitelson, J. S., J. Payne and L. Kim. 1992. *Bacillus thuringiensis*: insects and beyond. Biotech. 10:271-275.

Georghion, G. P. and M. C. Wirth. 1997. Influence of single versus multiple toxins of *Bacillus thuringiensis* subsp. *israelensis* on the development of resistance in the mosquito *Culex quinquefasciatus* (Diptera: Culicidae). Appl. Environ. Microbiol. 63:1095-1101.

Gill, S. S., E. A. Cowles and P. V. Pietrantonia. 1992. The mode of action of *Bacillusthuringiensis* endotoxins. Annu. Rev. Entomol. 37:615-636.

Hofte, H. and H. Whitely. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. 53:242-255.

Ibarra, E., M. Rincon, S. Orduz, D. Noriega, G. Benintede, R. Monerat, L. Rgis, C. Oliveira, H. Lanz, M. Rodriguez, J. Sanhez, G. Pena and A. Bravo. 2003. Diversity of *Bacillus thuringiensis* strains from Latin America with insecticidal activity against different mosquito species. App. Environ. Microbiol. 69:5269-5274.

Kronstad, J. W., H. E. Schnepf and H. R. Whitely. 1983. Diversity of locations for *Bacillus thuringiensis* crystal protein genes. J. Bacteriol. 154:419-428.

Lacey, L., A. R. Furtos, I. K. Kaya and P. Vail. 2001. Insects pathogens as biological control agents: Do they have a future? Biological Contr. 21:230-248.

Lamelli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Mohsen, Z. H., M. A. K. Ibrahim and N. S. Aujadooa. 1986. Isolation of spore-forming bacilli from mosquitoes in natural breeding habitats in Iraq. Entomophaga. 31(2):191-196.

Moraga, E., E. Tovar, P. Garcia and C. Alvarez. 2004. Isolation, geographical diversity and insecticidal activity of *Bacillus thuringiensis* from soil in Spain. Microbiol. Res. 159:59-71.

Rampersad, J., A. Khan and D. Ammons. 2002. Usefulness of Staining Parasporal Bodies when Screening for *Bacillus thuringiensis*. J. Invertebr. Pathol. 79:203-4.

Schnepf, E., N. Crickmore, J. Van Rie, D. L. Ereclus, J. Baum, J. Feitelson, D. R. Zeiglerand D. I. Dean. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. 62:775-806.

Service, M. W. 1986. Blood-sucking insects: Vectors of disease, Edward Arnold, London.

Siegel, J. P. 2001. A review of the mammalian safety of *Bacillus thuringiensis* based insecticides. J. Invertebr. Pathol. 77:13-21.

Thomas, W. E. and D. J. Ellar. 1983. Mechanism of action of *Bacillus thuringiensis* var. *israelensis* insecticidal delta-endotoxin. FEBS Lett. 154:362-368.

Van Rie, J., Van Mellaert, H., and Peferoen, M. 1992. Mechanism of insect resistance to Bacillus thuringiensis in Plodia interpunctella and Plutella xylostella. In: Molecular mechanisms of insecticide resistance: diversity among insects, Mullin, C. A. and Scott, J. G., Eds., American Chemical Society, Washington, DC.

Xavier, R., J. Reena and S. Sreeramanan. 2007. Environmental distribution and diversity of insecticidal proteins of *Bacillus thuringiensis* Berliner. Malaysian J. Microb. 3(2):1-6.

Zhang, H., Z. Yu and W. Deng. 2000. Isolation, distribution and toxicity of *Bacillus thuringiensis* from warehouse in China. Crop Protect:19:449-454.