การผลิตแบคคิวโลไวรัสจากเซลล์แมลงในระดับโรงงานต้นแบบ เพื่อใช้ปราบแมลงศัตรูพืช

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บทคัดย่อ

แบคคิวโลไวรัสเป็นไวรัสก่อโรคในแมลงที่ถูกนำมาใช้ควบคุมแมลงศัตรูพืช เนื่องจากมีประสิทธิภาพในการฆ่าหนอนแมลงและยังปลอดภัยต่อมนุษย์และสิ่งแวดล้อม แบคคิวโลไวรัสชนิด Nucleopolyhedrovirus (NPV) เป็นกลุ่มที่ถูกนำมาใช้อย่างแพร่หลายเนื่องจากอนุภาคไวรัสถูกห่อหุ้มด้วยผลึกโปรตีนทำให้มีความคงทนในสภาวะต่างๆในสิ่งแวดล้อมได้ดี การผลิต NPV เพื่อใช้ในการปราบแมลงมี 2 วิธี คือการต่อเชื้อในตัวหนอน และในเซลล์เพาะเลี้ยงของแมลง ในการศึกษานี้ได้ทำการผลิต NPV โดยใช้เซลล์แมลง (*Heliothis zea* cell line) ในระดับการผลิต 20 ลิตร โดยใช้ถังหมักแบบ stirred-tank reactor และใช้อาหารเลี้ยงเซลล์สูตรผสม พบว่าสามารถผลิตผลึกไวรัสสูงสุด 7.8 x 10^6 ผลึกต่อ มล. และจากการนำผลึกไวรัสที่ได้มาทดสอบประสิทธิภาพในการฆ่าหนอน ในระบบที่มีการควบคุมในห้องปฏิบัติการและในแปลงอุ่นพบว่าผลึกไวรัสมีประสิทธิภาพสูงเทียบเท่ากับผลึกไวรัสที่ผลิตจากหนอนแมลงที่ผลิตโดยกรมวิชาการเกษตรซึ่งมีการส่งเสริมให้เกษตรกรใช้ในปัจจุบัน นอกจากนี้ยังได้ศึกษาประสิทธิภาพของผลึกไวรัสภายหลังการทำสูตรสำเร็จ และพบว่ามีประสิทธิภาพที่ดีเทียบเท่ากับผลึกไวรัสจากตัวหนอนเช่นกัน ดังนั้นการใช้เซลล์เพาะเลี้ยงของแมลงจึงเป็นอีกทางเลือกหนึ่งที่เหมาะสมในการผลิต NPV เพื่อใช้ควบคุมแมลงศัตรูพืชเนื่องจากผลึกไวรัสที่ได้มีประสิทธิภาพและสามารถขยายขนาดการเพาะเลี้ยงได้ในระดับโรงงานต้นแบบ

คำสำคัญ : แบคคิวโลไวรัส / ผลึกไวรัส / NPV / เซลล์เพาะเลี้ยงของแมลง / *Helicoverpa armigera* / *Heliothis zea* / โรงงานต้นแบบ

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Pilot scale production of Baculovirus using insect cell culture for biopesticides

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Abstract

Baculoviruses are insect specific viruses which have been used as biopesticide for many years because of their effectiveness in insect control and safety for human and environment. The nucleopolyhedroviruses (NPVs) are the most widely used baculoviruses since the viruses are occluded in the polyhedral inclusion body which stabilizes them in the environment. There are 2 methods for NPV production, in insect larvae and insect cell culture. In this study, NPV production by insect cell culture (*Heliothis zea* cell line) in a stirred-tank reactor (20 L working volume) was investigated. Using medium mixtures developed in our laboratory, maximum yield at 7.8 x 10^6 BIPs/ml was obtained. The NPVs were tested for the biopesticide efficacy both in the control environment, in the laboratory, and in the field. It was found that the NPVs produced in this work were as potent as the NPVs produced from insect larvae by the Department of Agriculture which are currently distributed to the farmers. The formulated NPVs were also found to be efficacious. Thus, insect cell culture is a suitable host for NPV production. Not only the active NPV are produced, pilot scale production is also possible.

Keywords : Baculovirus / Polyhedral Inclusion Body / NPVs / Insect Cell Culture / *Helicoverpa armigera* / *Heliothis zea* / Pilot Scale

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1. Introduction

Baculoviruses are a diverse group of viruses with double-stranded DNA that is pathogenic for invertebrates [1]. The baculoviruses infect primary insects in the order Lepidoptera (e.g. moths and butterflies). The effectiveness of baculoviruses, and in particular nucleopolyhedroviruses (NPVs), for control of insect pests has been known for many years. Baculovirus NPV exists in two forms, a polyhedra-derived virus (PDV) and budded virus. The PDV are occluded within a protein crystalline matrix (Fig.1), a viral gene product called **'polyhedrin'**, which stabilizes the viral particles in the environment. Infection occurs when insect larvae ingest food contaminated with PDV. The alkaline nature in the insect midgut region results in dissolution of the crystalline polyhedrin matrix protein, releasing the PDV and allowing infection of the midgut epithelial cells. The PDV is however not very infectious to cultured cells. The budded virus, on the other hand, is enveloped nucleocapsids that are highly infectious to cultured insect cells but not stable in the environment [2, 3].

The baculoviruses are also known to be safe for human, other vertebrates and plants. These have made the NPV baculovirus a well known biopesticide for insect pest control. Most commercial productions, to date, have been done using *in vivo* methods. Thousands of larvae are required to prepare sufficient virus particles and this has made many problems for the quality control of commercial products. The viral product may contain many bacteria and other microorganisms, as well as insect proteins and cuticle. Mass rearing of insects, inoculation and harvesting of larvae are labor intensive. The most important disadvantage of *in vivo* production is that it is difficult to scale up economically. Production of baculovirus NPVs using insect cell culture is therefore a promising alternative strategy. As cell culture is operated under sterile condition, a far cleaner product is obtained. This makes quality control for certification of a viral product much easier. Furthermore, large-scale production can be easily performed in a bioreactor.

Fig.1 A) Electron micrograph of polyhedral inclusion bodies (PIBs) formed by NPV, which are stable in the environment.

B) PIB in the nucleus of the HaNPV-infected insect cell.

2. Materials and Methods

2.1 Cell line

Heliothis zea (Hz) cells were grown in 50% MM8 (a mixture of commercial media; 37.5% TC100, 12.5% SF900II and 50% minerals) supplemented with 5% fetal calf serum. The cells were cultured at 27 °C in 250-ml shake flask on an orbital shaker rotating at 120 rpm. When cell density reached 1-2 x 10^6 cells/ml, subculturing was performed (to 3 x 10^5 cells/ml).

2.2 Baculovirus isolation

The NPV was isolated from Nakhon Sawan, Thailand. Hemolymph was collected from infected *Heliocoverpa armigera* larvae after 72 hours post infection and infected to Hz cells. The infectious medium containing budded virus was collected and purified by plaque purification [4]. The purified isolate was amplified by infecting to Hz cells. The infectious medium was collected and the virus titer was determined by plaque assay [4].

2.3 Virus stock

Hz cells were infected with the plaque purified isolate of Thai HaNPV at a multiplicity of infection (moi) of 0.1 PFU/cell when cells were in the early exponential growth phase. The culture supernatant was collected at 4 days post-infection and used as a virus stock.

2.4 Production of NPV in a stirred tank reactor

Hz cells were seeded into 20-L 50% MM8 supplemented with 5% fetal calf serum at cell density of 3 x 10^5 cells/ml and cultured at 27 °C. The culture was performed in a 50-L stirred tank reactor with 30-70% DO air saturation and agitated at 50 rpm. After 2 days, the cells density reached 1 x 10^6 cells/ml and then the virus stock was added at an moi of 0.1 or 0.3. The infected cells were harvested at 7 days post infection by centrifugation at 5,000 rpm.

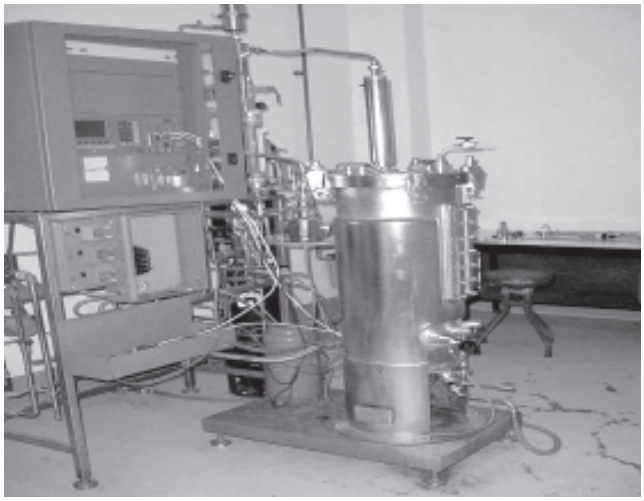

Fig. 2 A 50-L stirred-tank reactor for NPV production.

2.5 NPV preparation

The NPV infected insect cells were collected by centrifugation. The cell pellet was resuspended in distilled water and homogenized twice (each for 1 min) to release the PIB from the cells. The homogenized cells were centrifuged and the PIB pellet was resuspended in distilled water, counted and various concentrations were prepared in distilled water for bioassay.

2.6 NPV formulation

The HaNPV was formulated using the recipe from the Department of Agriculture (DOA). Ten milliliters of HaNPV suspension (7 x 10^8 PIBs/ml) was mixed with 15 ml Glycerine, 250 ml Emulsogen ELand 2 g of Triton X-100. The virus suspension was then tested for biopesticide efficacy as described in 2.7 A.

2.7 Bioassay

A) Laboratory assay

The NPV was tested for its efficacy under the controlled environment in laboratory. Thirty microlitres of HaNPV suspensions were dispensed onto the surface of artificial diet in a plastic cup. A control cup received the same volume of distilled water. A *H. armigera* larva was placed into each cup. Ten replicates/treatment were performed with ten larvae/replicate. Larval mortality was recorded daily for 7 days. Corrected mortality data were analyzed using Abbott's formula.

B) Field assay

The assay was performed at vineyard, Sampran, Nakhon Pathom. The HaNPV at the concentration of 3 x 10^6 PIBs/ml were prepared in distilled water. The virus suspension was sprayed onto the grape bunches both at the front and the back and left for 20 min to dry. Two insect larvae were placed on the treated bunch and then covered with a nylon bag (6 x 8 inches) sealed with an iron strip to retain the larvae inside the bag. Four replicates were performed and 5 grape bunches (with 2 larvae/bunch) were tested for each replicate. Larval numbers were recorded after 3 days and at final day (day 7).

3. Results and Discussion

1) HaNPV production

Production of HaNPV in 20-L insect cell culture was performed. For large-scale production, large volumes of virus inocula are required. It is therefore practical to infect the cells at low moi so that minimal virus stock is needed to be prepared. When low moi is used, the viral yields depend on the accumulation of viral products from multiple infections since some non-infected cell from the first infection continue to multiply and grow for the second round and the next infections [5]. The amount of initial inoculation therefore plays a role for the viral production.

Two concentrations of HaNPV (moi of 0.1 and 0.3) were tested for large scale production as suggested by previous work performed in shake flasks, when exponentially growing cells at cell density of 1 x 10^6 cells/ml were infected with various moi (0.01, 0.1, 0.2, 0.3, 0.5, and 1). Maximum HaNPV yield was obtained when moi at least 0.3 was used (data not shown). As shown in Table 1, HaNPV production of moi 0.3 was twice higher than that of moi 0.1. This was possibly due to more virus at moi 0.3 were available to infect into the insect cells than the moi of 0.1. This result was in agreed with the result from shake flask and as suggested by Weiss *et al.* [6]. Use of virus inoculum at moi 0.3 was considered economical for large scale production since small volume of virus stock will be used. The maximum yield (7.8 x 10^6 PIBs/ml) obtained from this work was lower than the previous work carried out by Weiss *et al.* where 5 x 10^7 PIBs/ml production was reported [6]. This possibly due to nutrient limitation since the medium used in this work was developed for insect cell growth not for the viral production. The limiting factors in the culture medium during viral infection are now being investigated to maximize the viral yields.

Table 1 Production of HaNPV from insect cell culture in 50-L stirred tank reactor (20 L working volume)

MOI	Viral Yield
MOI 0.1	4.3 x 10^6 PIBs/ml
MOI 0.3	7.8 x 10^6 PIBs/ml

PIBs = polyhedral inclusion bodies.

2) HaNPV as biopesticide

Non formulated HaNPV

A) Laboratory assay

The Thai isolate HaNPV produced from insect cell culture in this work was tested for its biopesticide efficacy compared to the HaNPV produced from insect larvae by the DOA in the control environment. Table 2 shows that biopesticide activity of the HaNPV produced by insect cell culture laboratory, King Mongkut's University of Technology Thonburi (KMUTT) was as efficacious as the HaNPV from DOA, which is now a commercial product. At lower number of HaNPV (2.5 x 10^5 PIBs/ml), higher% larval mortality caused by HaNPV, KMUTT was also obtained. This demonstrated that insect cell culture is suitable as an alternative method for production of active HaNPV to be used as a biopesticide.

Table 2 Efficacy of HaNPV produced from insect cell culture (HaNPV, KMUTT) and from insect larvae (HaNPV, DOA) against the *H. armigera* larvae

Sample	Total larvae/ treatment	2.5 x 10^5 PIBs/ml		1.2 x 10^6 PIBs/ml		Control (dH_2O)	
		dead no.	% mortality*	dead no.	% mortality	dead no.	% mortality
HaNPV (KMUTT)	100	80	79.79	75	74.74	1	0
HaNPV (DOA)	100	51	50.50	75	74.74	0	0

*Corrected % mortality by Abbott's formula = $\frac{(P-C) \times 100}{100-C}$ P = % mortality of treatment, C = % mortality of control

B) Field assay

Biopesticide efficacy of the HaNPV was examined in the open environment as shown in Table 3. The grape treated with HaNPV was better protected from the larvae when compared to the control where 70% damage of grape was shown. More damage of grapes was seen on the sample treated with the HaNPV (KMUTT) compared to the HaNPV (DOA). The loss larvae may contribute to some of the damages. This study demonstrated the potential of HaNPV as a biopesticide to reduce damage of the economically important plant such as grape from the pest.

Table 3 Efficacy of HaNPV produced from insect cell culture (HaNPV, KMUTT) and from insect larvae (HaNPV, DOA) against the *H. armigera* larvae on grape bunches

Samples	Total larvae	Lost larvae	Dead larvae	% Damage of grape bunches
Control (dH_2O)	40	5	2	70
HaNPV (KMUTT)	40	12	28	14
HaNPV (DOA)	40	5	35	5

Formulated HaNPV

The biopesticide efficacy of HaNPV was evaluated after formulation as flowable liquid product as shown in Table 4. Both local made HaNPV, KMUTT and HaNPV, DOA had higher efficacy for killing the *H. armigera* insect larvae (in the laboratory assay) than the imported product (Gemstar). The formulated HaNPV was also found to be more efficacious than the non-formulated virus. Only 75% mortality was observed from both non-formulated HaNPV at the concentration of 1.2×10^6 PIBs/ml as shown in Table 2 whereas higher than 90% mortality were shown by the two formulated HaNPVs. The chemicals added into the product probably enhance the efficacy of HaNPV from both sources.

Table 4 Efficacy of the formulated flowable HaNPV products

Samples	% mortality[#]
HaNPV (KMUTT)	91.66
HaNPV (DOA)	93.75
HaNPV (Gemstar)*	85.42

% Mortality when 1×10^6 PIBs/ml was used.

*Gemstar: a commercial HaNPV imported product.

4. Conclusions

Pilot scale production of the Thai isolate HaNPV was performed in a 50-L stirred tank reactor (20 L working volume). The HaNPV products from this production (KMUTT) were tested for their biopesticide efficacy and found to be as efficacious as the HaNPV produced from insect larvae by DOA. This work demonstrates the potential use of insect cell culture as an effective method for large-scale production of baculovirus for biopesticide.

5. Acknowledgement

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