Optimization of Propagation of *Anagrapha falcifera* Nuclear Polyhedrosis Virus in *Spodoptera frugiperda* 21 Cells

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Abstract Propagation of *Anagrapha falcifera* nuclear polyhedrosis virus (AfNPV) was investigated using well-plates and split-flow air-lift bioreactors. In well-plate experiments, the effects of pH, cell density at a point of infection, serum concentration, DEAE-dextran, and lipid on virus propagation were all closely examined. The AfNPV titer in well-plates was optimal at pH 6.8 and 3×10^6 cells/cm^2. The virus titer was not dramatically affected when the fetal bovine serum concentration was reduced from 10% to 5%. The addition of cholesterol at AfNPV infection of Sf21 cells enhanced the virus titer, whereas the addition of DEAE-dextran did not improve the titer. The AfNPV titer (3.8×10^7 TCID$_{50}$/ml) at optimized conditions for well-plate experiments was 2.5-fold higher than for the control. In bioreactor experiments, the AfNPV titer showed its maximum level at air flow rates of 20–40 ml/min. In a split-flow air-lift bioreactor, AfNPV titer (2.3×10^7 TCID$_{50}$/ml) was 1.5-fold higher than the control when the culture was at pH 6.8 and supplemented with 0.34 mM cholesterol.

Key words: *Anagrapha falcifera* nuclear polyhedrosis virus, split-flow air-lift bioreactor, pH, cholesterol

Insect viruses have received considerable amount of attention because they have shown a potential for being safe and effective biological control agents. The nuclear polyhedrosis viruses (NPVs) are an important subgroup of pathogenic baculoviruses which may prove to be valuable as insect control agents [23]. Recently, the celery looper virus, known as *Anagrapha falcifera* nuclear polyhedrosis virus (AfNPV), has been isolated [1]. It is a rod-shaped

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virus with a double-stranded and circular genome [2]. The use of AfNPV as a biological insecticide is highly probable [8] since the range of hosts of this virus is relatively wide.

Commercial production of NPVs is currently performed by viral propagation in infected insect larvae. However, the development of an adequate technology to produce AfNPV in infected insect cell cultures would drastically improve quality and safety. Batch propagation of baculoviruses in infected cell cultures has been shown to be dependent on cell culture conditions along with infection conditions [4, 6, 10, 12, 15, 20-22] as well. Despite of many reports on viral propagation of other baculoviruses [6, 7, 10-13, 22], researches on optimization of AfNPV propagation is quite scarce.

Improvement in NPV propagation can be achieved by carrying out various cultivation strategies. Manipulation of the cultural environment is one of the basic strategies to improve NPV propagation. Key factors for NPV propagation are cell concentration, pH level at infection, FBS, lipid, and DEAE-dextran concentration. In this work, we investigated optimum conditions for the propagation of AfNPV using well-plate and split-flow air-lift bioreactor.

The *Spodoptera frugiperda* 21 cells (Sf21) were obtained from L. K. Miller (University of Georgia, Athens, GA, U.S.A.). Insect cell culture medium was TNM-FH (Sigma Chemical Co., St. Louis, MO, U.S.A.) which contained gentamycin sulfate (50 μg/ml), fungizone (25 μg/ml), sodium bicarbonate (0.35 μg/ml), and 10% FBS [17]. For the infection medium, TNM-FH was supplemented as follows: gentamycin sulfate (50 μg/ml), fungizone (2.5 μg/ml), sodium bicarbonate (0.35 μg/ml), and 5% FBS. Unless otherwise specified, the pH of all media was adjusted to 6.2, and sterilized with cellulose nitrate filter (0.2 μm) (GelmanSciences, Ann Arbor, MI, U.S.A.). *Anagrapha falcifera* nuclear polyhedrosis virus was a gift from J. S. Manning (University of California, Davis, CA, U.S.A).

The glass split-flow air-lift bioreactor with a ratio of riser cross-sectional area to downcomer cross-sectional area of 1, and a ratio of height to diameter of 4.4 was identical to that described by Chung et al. [3]. A 200 mesh stainless steel cloth was placed at the bottom of the downcomer to support the packing in the column. Sf21 cell was cultivated in 3 mm-diameter glass beads located in the downcomer. Air (at flow rates of 20, 40, and 60 ml/min) supplied to the riser section provided the motive force for medium circulation and aeration. The air-flow rate was monitored and controlled by an air-flow meter (Cole-Parmer Instrument Company, Chicago, IL, U.S.A.). The pH of the media was adjusted to 6.2, unless otherwise specified. The system was kept at 27°C in an incubator.

Sf21 cells grown from T-flasks were seeded into 24-well-plates and allowed to be attached for 1 h. AfNPV was added at a multiplicity of infection (MOI) of 1. Then, the medium containing virus was removed and replaced with the infection medium, and the cells in well-plates were incubated at 27°C. For specific supplementation, DEAE-dextran (M.W. 500,000), cholesterol, tocopherol, and β-estradiol were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Lipid components were used at the 0.34 mM concentration which was previously determined [14]. All stocks of the medium additives were filter-sterilized. For well-plate experiments, initial cell concentration was 3×10^5 cells/cm^2, unless otherwise specified. In the case of the bioreactor, an initial cell concentration was 1×10^5 cells/ml in which AfNPV was added to a MOI of 1, unless otherwise specified. Samples were taken 4 days post-infection to determine virus titers.

Viable cell concentration was determined by a hemocytometer and the titer of AfNPV was measured by TCID$_{50}$ which was determined by defining the dilution of virus required to infect 50% of the cell culture inoculated [19]. Relative virus titer was calculated by dividing each virus titer by a maximum virus titer. All the data were represented as averages of duplicate experiments.

The effects of initial cell concentration on AfNPV propagation were tested in well-plates seeded with concentrations of Sf21 ranging from 1×10^5 to 3.0×10^6 cells/well. The virus titer increased up to 3×10^5 cells/well and then remained relatively constant (6-7×10^7 TCID$_{50}$/ml) (Fig. 1). Unlike *Trichoplusia ni* 5B1-4 cells [2], sensitivity to contact inhibition did not appear at 1-3×10^6 cells/well. It suggests that Sf21 cells can grow in a suspension culture, in contrast to *T. ni* 5B1-4 cells.

The optimum pH for insect cell growth may vary from species to species. However, *Spodoptera frugiperda* 21 cells are usually grown at pH 6.2, although no experimental justification has been given [16]. Therefore, experiments were carried out to find the effect of pH at the time of infection on virus propagation (Fig. 2). The titer was shown to be maximum at pH 6.8, showing 40% improvement compared to the titer at pH 6.2.
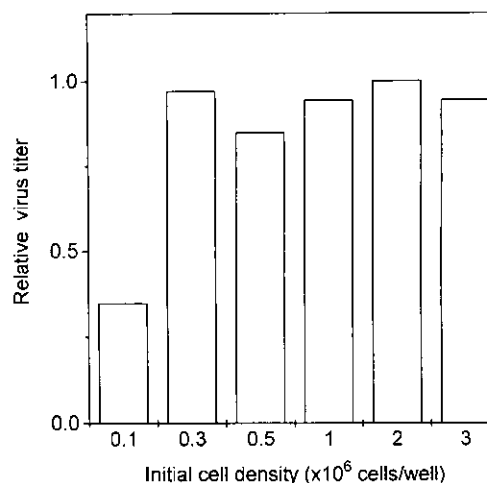

Fig. 1. Effect of initial cell density on AfNPV titers in well-plates.

Fetal bovine serum is a costly component in the medium for virus propagation. To determine to what extent the FBS concentration could be reduced, the effect of serum concentration on AfNPV propagation was tested in a well-plate (Fig. 3). A severe decrease in virus titer was observed as the FBS concentration was reduced to 2%. However, the maximum virus titer was not dramatically affected when the FBS concentration was reduced from 10% to 5%.

Figure 4 illustrates the effect of DEAE-dextran on the propagation of AfNPV. DEAE-dextran is known to promote the uptake of protein or polynucleotide into the cells and to increase the infection of virus into the cells [9]. We found that AfNPV propagation in DEAE-dextran at a level of 10-20 μg/well increased the AfNPV titer slightly; however, DEAE-dextran at higher than 30 μg/well decreased the titer.

Addition of lipids had a positive effect on enhancing virus titers (Fig. 5). In fact, cholesterol was found to be the
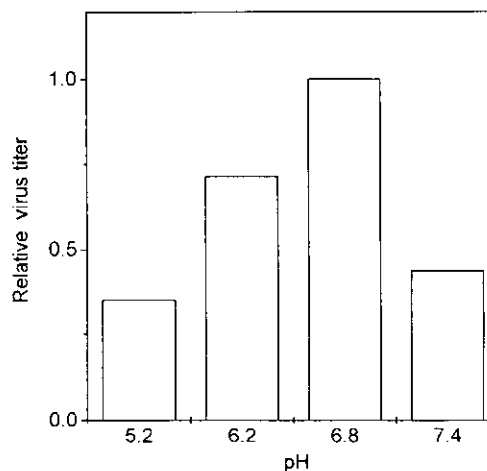

Fig. 2. Effect of pH on AfNPV titers in well-plates.
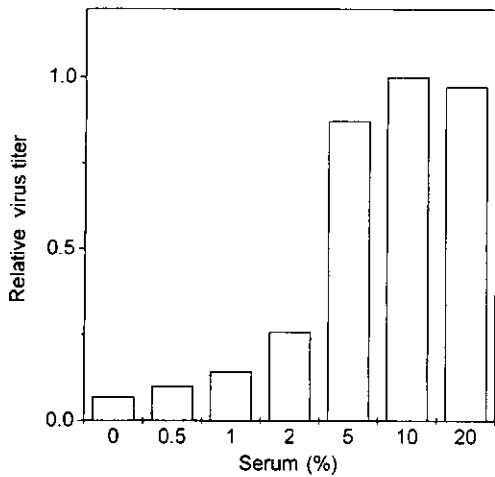
Fig. 3. Effect of serum concentration on AfNPV titers in well-plates.

best among the lipids tested. It increased the virus titer 3.4-fold, compared to the lipid-free control. Since cholesterol plays an important role in stimulating cell growth [5], and is also involved in the regulation of membrane fluidity [18], we speculate that the effect of cholesterol in raising the virus titer is due to the enhancement of membrane fluidity.

From this study, optimal cultural conditions such as initial cell concentration, pH at infection, FBS concentration, and lipid on AfNPV propagation in 24-well-plates were determined to be as follows: initial cell concentration of 1×10^6 cells/well, pH 6.8, 5% FBS, and cholesterol of 0.34 mM, respectively. Table 1 shows the results of the experiment conducted at the above stated optimum conditions. The virus titer was increased 2.5 times to 3.8×10^7 TCID$_{50}$/ml, compared to the control (1.5×10^7 TCID$_{50}$/ml). The treatment at optimum conditions as specified above gives a
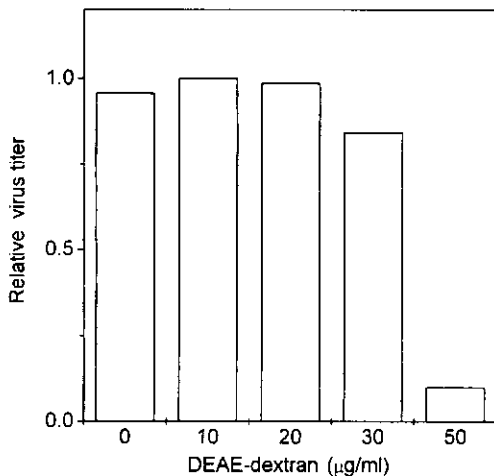

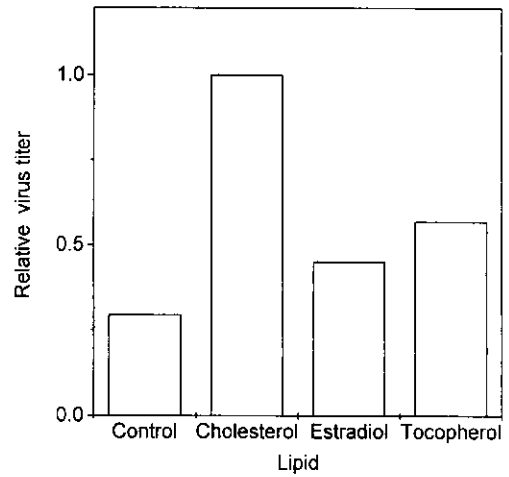**Fig. 4.** Effect of DEAE-dextran on AfNPV titers in well-plates.

Fig. 5. Effect of lipid on AfNPV propagation in well-plates.

somewhat lower titer than expected, since the use of cholesterol alone gives about a 3.4-fold enhancement.

A set of experiments was carried out to propagate AfNPV in a spilt-flow air-lift bioreactor. As shown in Table 2, air flow rates of 20, 40, and 60 ml/min were employed and the results were compared to those of well-plate experiments. The highest virus titer was obtained at 20–40 ml/min. This indicates that optimum conditions for aeration exist in the split-flow bioreactor. Within this range of air-flow rate, the flow regime in downcomer would be in the laminar flow region, since our previous calculation indicated that its Reynolds number was as low as 10 or less than that at these flow rates in this bioreactor [3]. The effect of MOI values on AfNPV propagation was also examined at the air-flow rate of 20 ml/min. Figure 6 shows viral propagation in the split-flow air-lift bioreactor after infection at MOIs of 0.1, 1, and 5. The titer at an MOI of 1 was higher than that obtained at MOIs of 0.1 and 5.

Table 1. An optimal condition for virus propagation in a 24-well-plate.

pH	Cholesterol	Virus titer at 4 days postinfection ($\times 10^7$ TCID$_{50}$/ml)
6.2	–	1.5
6.8	+	3.8

*: +, with supplementation; –, without supplementation.

Table 2. Virus propagation in a split-flow air-lift bioreactor.

Air-flow rate (ml/min)	pH	Cholesterol	Virus titer ($\times 10^7$ TCID$_{50}$/ml)
20	6.2	–	1.50
40	6.2	–	1.40
60	6.2	–	0.51
20	6.8	+	2.30

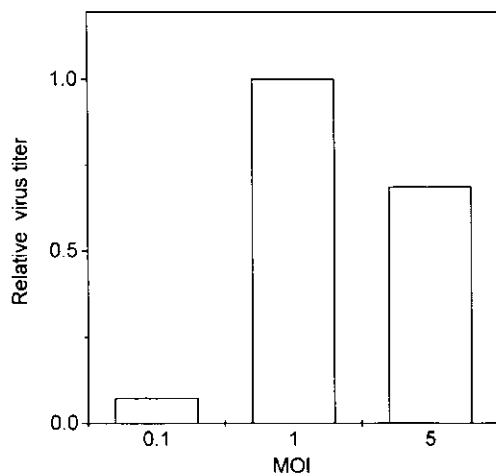*: +, with supplementation; –, without supplementation.

Fig. 6. Effect of MOI on AfNPV propagation in a split-flow air-lift bioreactor.

Optimum conditions derived from the well-plate study were used to propagate AfNPV in a split-flow air-flow bioreactor. As shown in Table 2, the virus titer was increased to 2.3×10^7 $TCID_{50}$/ml which was 1.5 times higher than the control (1.5×10^7 $TCID_{50}$/ml). This is slightly lower than the enhancement obtained in the well-plate (See Table 1) which was 2.5-fold higher. Nevertheless, the split-flow air-lift system gave $0.5-1.5 \times 10^7$ $TCID_{50}$/ml. Our results indicate that a split-flow air-lift bioreactor was appropriate in the propagation of AfNPV virus, and scale-up of this system is easy and aeration is possible by sparging without direct exposure of air bubbles to the cells. Therefore, the split-flow air-lift bioreactor can be applied not only to the propagation of insect cells but also to that of other animal cells.

Acknowledgments

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