Kinetic Analysis of *in vitro* Production of Wild-Type *Spodoptera frugiperda* Nucleopolyhedrovirus

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ABSTRACT

In this study, the kinetic behavior of Sf9 and Sf21 cells used in the production of a baculovirus biopesticide to control the pest of corn Spodoptera frugiperda *was analyzed. Kinetic variables such as maximum specific growth rate, cell productivity, mean rate of infection, as well as the mean rate of occlusion body production were determined during the infection of these cell-lines with the extracellular virus of the* S. frugiperda *nucleopolyhedrovirus (SfMNPV). The Sf9 cell-line resulted in better viral production results (5.0 x 10^8 OB/mL) than the Sf21 cell-line (2.5 x 10^8 OB/mL).*

Key words: Baculovirus, *in vitro* production, kinetic analysis, SfMNPV

INTRODUCTION

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith, 1977) (Lepidoptera: Noctuidae), is the main pest of corn on the entire American continent, being responsible for incalculable crop losses. It is currently controlled by chemical pesticides that generally cause environmental damage and health risks. A large problem with this pest is its development into pesticide-resistant populations (Cruz, 2002). As an alternative, biopesticides have been produced that can be applied to various agricultural crops. For this reason, biocontrol has been the focus of innumerable studies, with Brazil leading the research on the use of baculoviruses as biological control agents.

Baculoviruses are host-specific viral biopesticides that are important agents of biological pest control

(Martignoni, 1984; Moscardi, 1999; Moscardi and Souza, 2002). They comprise the largest group of insect viruses and their use in agroecosystems causes minimal disturbance to beneficial arthropods and minimal ecological damage (Szewczyk et al., 2006). They are protected inside protein crystals (occlusion bodies), which permit the formulation of easily-applied biopesticides that are more economical and safer than chemical insecticides. So far, there have been no field reports of insects developing acquired resistance to these viruses (Moscardi, 1999).

Animal cell culture is important because of the possibility of bioproduct synthesis, which is devoted mainly to human and animal health. Products include viral vaccines, monoclonal antibodies, enzymes, hormones and biopesticides. In particular, insect cells play an important role in

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the *in vitro* production of viral biopesticides (Augusto and Oliveira, 2001).

Processes involving the insect cells differ from those using microorganisms, because the former uses the cells extracted from insect tissue and adapted to *in vitro* culture (Augusto and Oliveira, 2001). Because of advances in cell culture, the production of baculoviruses in insect cells can be achieved in different growth systems, from monolayer static culture to suspension culture using Erlenmeyer flasks, spinners or bioreactors. Most insect cells can be cultured at 25-30°C (Agathos et al., 1990); however, the optimal temperature during cell growth and the infection of Sf9 and Sf21 cell lines is 28°C (King and Possee, 1992).

Suspension cultures favor the study of kinetic variables of cell growth and protein production. The cell density is estimated frequently during the entire culture period, since the estimation of specific growth and production rates depends on this variable (Hiss, 2001). The cells used in this study were isolated from the embryonic tissues of the fall armyworm *(S. frugiperda)*, and are known as Sf9 and Sf21 cell lines. These cells are easily adapted to suspension culture (King and Possee, 1992). The cell line used may compromise *in vitro* production of the baculovirus, by displaying different permissivity of virus replication (Bilimoria et al, 1992). Therefore, even cells permissive to replication can result in different virus production levels depending on the cell line used.

The production of a baculovirus involves the infection of cells permissive to the virus. The *in vitro* infection process is initiated with extracellular budded virus (BV), which is obtained from the hemolymph of infected insects or from the supernatant of infected cells. When infected, insect cells produce more BV, as well as the occluded form of the virus, Occlusion Bodies (OB) (King and Possee, 1992). OBs are the biopesticidal form of the virus in the field. In addition, in vitro infection can be initiated with Occlusion Derived Virus (ODV) extracted from OB (Lynn, 1994).

The aim of this work was to study the kinetics of cellular growth and of baculovirus production, by infecting suspension-grown Sf21 and Sf9 cells with *Spodoptera frugiperda* nucleopolyhedrovirus (SfMNPV, isolate 18).

MATERIALS AND METHODS

Cells
The insect cell lines (Sf9 and Sf21) used were kindly provided by Embrapa Genetic Resources and Biotechnology, and were originally sourced from the American Type Culture Collection (ATCC, MD)

Virus
The virus used was *Spodoptera frugiperda* nucleopolyhedrovirus (SfMNPV) isolate 18, which was isolated from corn fields and kindly donated by Dr. Fernando Valicente – Embrapa Corn and Sorghum.

Cell culture
Cells were adapted and cultured in shake flasks (Certomat MO II – B. Braun) at 120 rpm and 28°C using HyQ SFX-Insect™ (HyClone) medium supplemented with 5% (v/v) of fetal bovine serum (FBS) (Invitrogen). Cell subcultures were performed every 72 to 96 h to maintain them in the exponential growth phase. Cell concentration was estimated using a phase contrast microscope (Olympus, Japan) and a Neubauer hemocytometer (Bright-Line Hemocytometer, Sigma). The concentration of viable cells and viability were determined by the trypan blue exclusion technique (Sigma-Aldrich) at a final concentration of 0.1% (v/v). A 15% deviation was obtained by daily counting in triplicate of 200 cells on both sides of the hemocytometer (Nielsen et al., 1991).

In vitro infection with budded virus of SfMNPV
For kinetic analysis of the baculovirus, both Sf9 and Sf21 cells were infected with the Budded Virus (BV) of SfMNPV. BVs were obtained from a preliminary *in vitro* infection initiated with Occlusion-Derived Virus (ODV) liberated from caterpillar-produced Occlusion Bodies (OB) (Lynn, 1994). This initial infection was set up (in both Sf9 and Sf21 cells) to obtain the Passage 1 virus stock (containing BV), which was the recovered supernatant after centrifugation (1000xg for 10 min) of the infected cell culture. For Passage 2 virus infections, 3 mL of Passage 1 virus stock was used to infect 30 mL shaker cultures of Sf9 and Sf21 cells (respectively), at an initial cell density of 5 x 10^5 viable cells/mL. Non-infected cell culture controls were also set up at

the same cell density. The cell densities and growth rates were determined daily. At the end of 10 days post infection (dpi), OB production in infected cells was determined by OB counts using a Neubauer hemocytometer (Bright-Line Hemocytometer, Sigma). All the experiments were done in triplicate.

Kinetic variables

The maximum cell-specific growth rates of the control and infected cells (μ_x) were estimated by Neperian logarithmic linear fitting of cell density with cultivation time during exponential growth. The angular coefficient of this curve was the maximum specific growth itself. The mean infection rate (K_d) and mean OB production rate (K_P) were calculated by linear fitting of

experimental data during the exponential growth phase of infected cells.

RESULTS AND DISCUSSION

Fig. 1 shows the growth curve of Sf9 cells in HyQ SFX-Insect[TM] (HyClone) medium supplemented with 5% FBS for both infected and non-infected (control) cells. For the control culture, a maximum cell density (X_{max}) of 7.4 x 10^6 viable cells/mL was recorded after five days of culture and a cell productivity (P_x) of 0.77 x 10^6 cells/mL.d. was recorded over nine days of culture. The infected culture reached a maximum cell density (Xi_{max}) of 1.0 x 10^6 viable cells/mL at four days post infection (dpi) and a cell productivity (P_{xi}) of 0.068 x 10^6 cells/mL.d.
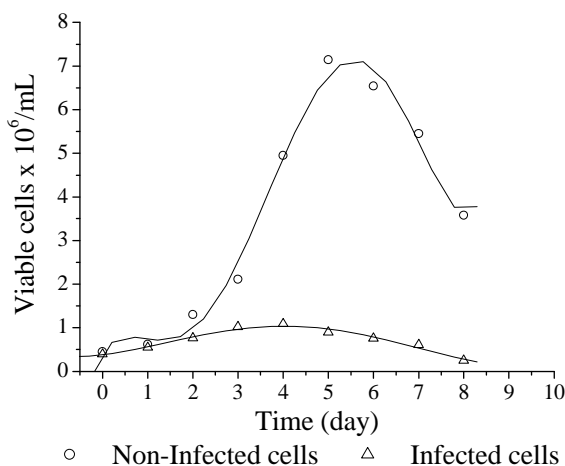

Figure 1 - Growth curve of non-infected and infected Sf9 cells.

The maximum specific growth rates of non-infected ($\mu_{x_{max}}$) and infected cells (μ_d)Sf9 cells were estimated by logarithmic transformation of the cell growth curve during the exponential growth phase (observed between 1 and 4 days of culture for the non- infected cells and from 0 to 3 days of culture for the infected cells). The estimations were 0.596 d^{-1} for $\mu_{x_{max}}$ and 0.321 d^{-1} for μ_d. The mean infection rate (K_d) for Sf9 cells was estimated at 0.196 cells/mL.d. The production of OB in Sf9 cells is depicted in Fig. 2. The maximum OB production was 5.0 x 10^8 OB/mL at

9 dpi and the mean OB production rate (K_p) was estimated at 11.070 OB/mL.d.
Fig. 3 shows the growth of non-infected and infected Sf21 cells, under the same culture conditions as those for Sf9 cells. For non-infected cells, the maximum cell density (X_{max}) was 10.0 x 10^6 viable cells/mL and the cell productivity (P_x) was 1.1 x 10^6 cells/mL.d. Infected cells had a maximum cell density (Xi_{max}) of 0.7 x 10^6 viable cells/mL and cell productivity (P_{xi}) of 0.024 x 10^6 cells/mL.d.
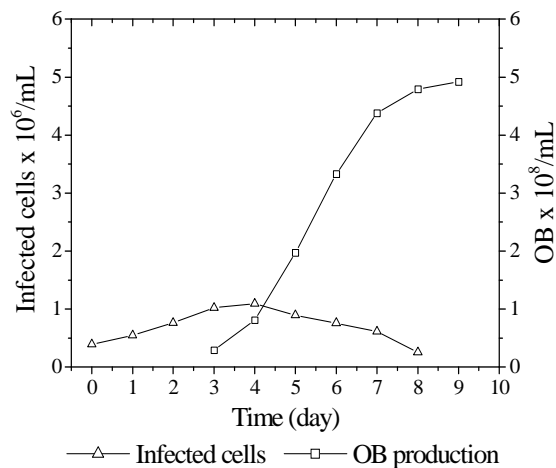
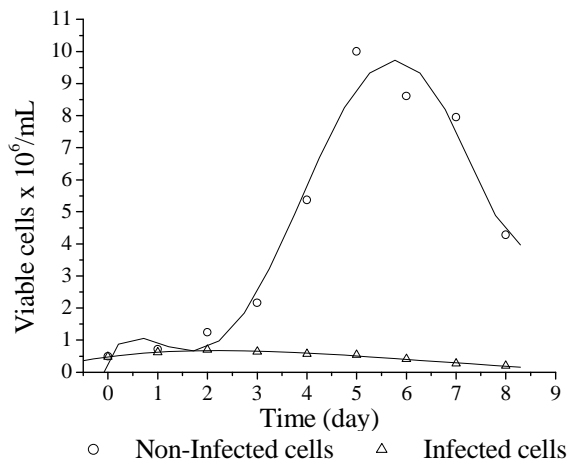**Figure 2 -** Growth curve of infected Sf9 cells and OB production curve.

Figure 3 - Growth curve of non-infected and infected Sf21 cells.

The maximum specific growth rates of non-infected ($\mu_{x_{max}}$) and infected (μ_d) Sf21 cells were calculated by logarithmic transformation of the cell growth curve during the exponential phase (observed between 2 and 5 days of culture for the non-infected cells, and from 0 to 2 dpi for the infected cells). The estimations were 0.718d^{-1} for

$\mu_{x_{max}}$ and 0.191d^{-1} for μ_d. The mean infection rate (K_d) for Sf21 cells was estimated at 0.087 cells/mL.d .

OB production in Sf21 cells is shown in Fig. 4. The maximum OB production was 2.5 x 10^8 OB/mL at 9 dpi, and the mean OB production rate (K_p) was 0.583 OB/mL.d.
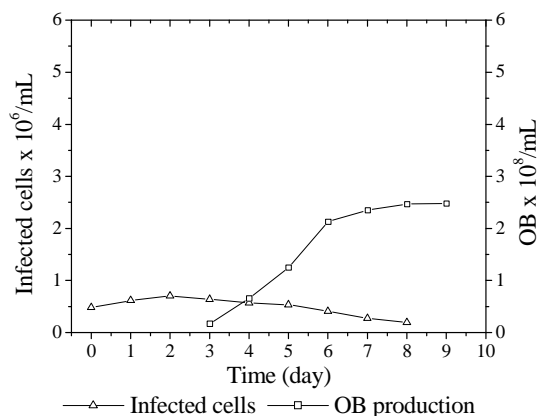
Figure 4 - Growth curve of infected Sf21 cells and OB production curve.

The infection kinetics of Sf9 and Sf21 cells (within 4 and 3 dpi, respectively) followed a simple zero-order linear kinetic model. The angular coefficient of the infected cell growth profile, fitted by a linear regression, was the proper velocity constant and defined as the mean infection rate (K_d). The rate of OB production (K_p) can be defined in a similar way. It would be useful to compare the kinetics of non-infected Sf9 and Sf21 cell growth in suspension cultures (HyQ SFX-Insect^{TM} (HyClone) medium) obtained from this work with those of other cell culture systems from the literature. Hild et al. (1992) showed that Sf9 cells grown in TC-100 medium supplemented with 5% (v/v) FBS reached a maximum density of between 2.9 and 3.8 x 10^6 cell/mL and a specific growth rate of 0.67 d^{-1}. Fernandes et al. (2005) showed that ST (*Swine testis*) cells grew to a maximum viable cell density of 1.6 x 10^6 cells/mL,

and exhibited a cell productivity of 0.34 x 10^6 cells/mL.d. Barral et al. (2005) demonstrated that S2 cells (*Drosophila melanogaster*) could be grown to a maximum cell density of 4.5 x 10^6 cells/mL, with a maximum growth rate (μ_{max}) of 0.68 d^{-1}) and a cell death rate (μ_d) of 2.8x10^3 d^{-1}.

The Sf9 and Sf21 cell lines in this work showed a higher level of cell production than the other cell lines mentioned above. However, the duration of the exponential growth phase and the specific growth rates were quite similar among the cell lines being compared (Table 1).

These findings showed that the values obtained in our study were consistent with literature data.

Table 2 summarizes the kinetic variables obtained from this work for infected Sf9 and Sf21 cells: including the specific infected growth rate, cell productivity, mean infection rate, and mean OB production rate.

Table 1 - Comparing the growth kinetics of non-infected Sf9 and Sf21 cells with those of other animal cells.

Kinetic Variables	Cells	
	Sf9	Sf21
$X_{initial}$ (cells.mL^{-1})	0.5x10^6	0.5x10^6
$X_{maximum}$ (cells.mL^{-1})	7.4x10^6	10.0x10^6
$\Delta X_{maximum}$ (cells.mL^{-1})	6.9x10^6	9.5x10^6
Duration exp. phase (d)	3.0	3.0
P_{Xmax} (cells.mL^{-1}.d^{-1})	0.77x10^6	1.1x10^6
$\mu_{Xmax.}$(d^{-1})	0.596	0.718
Kinetic Variables	**Cells**	
	ST	S2
$X_{initial}$ (cells.mL^{-1})	0.21x10^6	0.5x10^6
$X_{maximum}$ (cells.mL^{-1})	1.6x10^6	4.5x10^6
$\Delta X_{maximum}$ (cells.mL^{-1})	4.1x10^6	4.0x10^6
Duration exp. phase (d)	3.04	---
P_{Xmax} (cells.mL^{-1}.d^{-1})	0.34x10^6	---
$\mu_{Xmax.}$(d^{-1})	---	0.68

Table 2 - Kinetic results of infections with the SfMNPV virus in Sf9 and Sf21 cells.

Kinetic Variables	Cells	
	Sf9	Sf21
$Xi_{initial}$ (cells.mL^{-1})	0.39×10^6	0.48×10^6
$Xi_{maximum}$ (cells.mL^{-1})	1.0×10^6	0.7×10^6
$\Delta Xi_{maximum}$ (cells.mL^{-1})	0.61×10^6	0.22×10^6
P_{Ximax} (cells.mL^{-1}.d^{-1})	0.068×10^6	0.024×10^6
μ_d (d^{-1})	0.321	0.191
K_d (cells.mL^{-1}.d^{-1})	0.196	0.087
K_p (OB.mL^{-1}.d^{-1})	1.070	0.583

The specific growth rate of non-infected Sf21 cells ($\mu_{xmax} = 0.718$ d^{-1}) was around 20% higher than that of non-infected Sf9 cells ($\mu_{xmax} = 0.596$ d^{-1}). Similarly, the cell productivity of non-infected Sf21 cells was 43% higher than that of non-infected Sf9 cells ($P_{Xmax} = 0.77 \times 10^6$ cells/mL.d), showing that Sf21 cells were totally adapted to the culture conditions developed in this system.

The specific maximum growth rates (μ_{max}) for Sf9 and Sf21 cells were previously reported to be between $0.67 - 0.83$ d^{-1}, which corresponded to a duplication time of around $0.83 - 1.04$ d (Schaeger et al., 1993). Therefore, the maximum specific growth rates for Sf9 and Sf21 cells obtained from this work were in accordance with literature data.

Sf9 cells produced twice as many OBs (5×10^8 OB/mL) as did Sf21 cells (2.5×10^8 OB/mL), as shown in Figs. 2 and 4. This could also be demonstrated by the mean rate of product formation, where the rate of OB formation for Sf9 cells ($K_p = 1.070$ OB/mL.d) was 83% higher than that for Sf21 cells ($K_p = 0.583$ OB/mL.d). In addition, Sf9 cells exhibited greater infection efficiency than Sf21 cells, since the mean infection rate of Sf9 cells ($K_d = 0.196$ cells/mL.d) was 2.25 times higher than that of Sf21 cells ($K_d = 0.087$ cells/mL.d), under the same culture and infection conditions.

CONCLUSION

This work analyzed the infection kinetics of SfMNPV in both Sf9 and Sf21 cell-lines, which were propagated in suspension cultures (28°C and 120 rpm) using HyQ SFX-Insect™ (HyClone) medium supplemented with 5% (v/v) of FBS. Sf9 cells exhibited a higher mean infection rate ($K_d = 0.196$ cells/mL.d) than that of Sf21 cells ($K_d = 0.087$ cells/mL.d). In addition, Sf9 cells also supported a higher OB yield (5.0×10^8 OB/mL),

with a mean OB production rate (K_p) of 1.070 OB/mL.d.

RESUMO

Neste trabalho, analisou-se o comportamento cinético das células Sf9 e Sf21 utilizadas na produção de biopesticida para o controle de *Spodoptera frugiperda*. Variáveis cinéticas, como velocidade específica máxima de crescimento, produtividade em células, velocidade média de infecção e a velocidade média de produção de OB foram determinadas durante a infecção destas linhagens com o vírus extracelular do nucleopoliedrovirus de *S. frugiperda*. A linhagem Sf9 resultou em melhores resultados de produção do baculovírus (5×10^8 OB/mL), quando comparada à linhagem Sf21 (2.5×10^8 OB/mL) e outras linhagens da literatura.

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