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Production of occlusion bodies of *Anticarsia gemmatalis multiple nucleopolyhedrovirus* in serum-free suspension cultures of the saUFL-AG-286 cell line: Influence of infection conditions and statistical optimization

Gabriela A. Micheloud[a,b], Verónica V. Gioria[a,b], Gustavo Pérez[c], Juan D. Claus[a,b,*]

[a] *Laboratorios de Virología y Fermentaciones, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Argentina*
[b] *Instituto de Agrobiotecnología del Litoral-UNL-CONICET, Argentina*
[c] *INGAR-CONICET; (3000) Santa Fe, Argentina*

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A B S T R A C T

The influence of the conditions of infection on the yield of occlusion bodies (OBs) of the *Anticarsia gemmatalis multiple nucleopolyhedrovirus* (AgMNPV), produced in serum-free suspension cultures of saUFL-AG-286 cells, was investigated by two 2^2 full factorial experiments with centre point. Each experiment tested the effects of the initial cell density and the multiplicity of infection at two levels, in the four possible combinations of levels and conditions, plus a further combination with each condition set at the middle of its extreme levels. The yield of occlusion bodies proved to be sensitive to the modification of infection conditions. Maximum yield as high as 3×10^8 OBs mL^{-1} was attained provided that the maximum density of viable cells was in the range between 4 and 8×10^5 cells mL^{-1}. The optimum value of the maximum density of viable cells could be reached by the combination of several values of initial cell density and multiplicity of infection. A regression model was established and validated in order to optimize the infection conditions. These results demonstrate the importance of an adequate selection of infection conditions, and they could be useful in the development of a feasible *in vitro* process to produce the AgMNPV insecticide in a new serum-free medium.

1. Introduction

Anticarsia gemmatalis Hübner (Lepidoptera: Noctuidae) is one of the main plagues of soybean crops. This insect is controlled efficiently with OBs of an insecticide baculovirus, *Anticarsia gemmatalis multiple nucleopolyhedrovirus* (Carner and Turnipseed, 1977; Moscardi, 1999). This insecticide is produced currently in infected larvae of the target insect, either in parcels of soybean fields or in biofactories (Szewczyk et al., 2006). This technology has proved to be robust and competitive economically when compared with chemical control but a growing demand and scaling-up limitations have stimulated an interest in developing alternative processes based on viral propagation in insect cell cultures (Rodas et al., 2005). Several reports have described the production of AgMNPV OBs in insect cell cultures (Batista et al., 2005; Castro et al., 1997; Castro et al., 2006; Claus et al., 1993; Gioria et al., 2006; Grasela and McIntosh, 1998; Rodas et al., 2005; Visnovsky and Claus, 1994; Zhang et al., 2005), but either the production conditions or the yields proved to be incompatible with the development of an economically feasible production process. One of the main requisites to achieve this goal is the selection of an optimized strategy for infecting cell cultures.

The strategy of baculovirus production in a batch culture of insect cells is determined mostly by the selection of the multiplicity of infection (MOI) and the initial cell density (ICD). The MOI is defined as the number of infectious units per cell that are added at the time of infection. Both the fraction of the cellular population infected initially and the kinetics of culture infection are related to the multiplicity of infection. On the other hand, in a baculovirus-infected batch culture of insect cells, the selection of the initial cell density determines the composition of the culture medium at the time of infection as well as the physiological state of the cellular biomass. These two factors interact with each other and can be manipulated to optimize the virus yields (Licari and Bailey, 1992). Although the influence of the MOI and ICD has been well established for a variety of baculovirus-insect cell systems destined for the production of either recombinant proteins or viral OBs, the optimal combination of these two conditions must be established for every system because it depends on both the characteristics of the

* Corresponding autor at: Laboratorio de Virología, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, CC 242, (3000) Santa Fe, República Argentina. Tel.: +54 342 4575216x118; fax: +54 342 4575216x118.
E-mail addresses: jclaus@fbcb.unl.edu.ar, juandanielclaus@yahoo.com.ar (J.D. Claus).

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insect cell line and the nutritional capability of the culture medium employed in a given baculovirus production process (Wong et al., 1996; Yang et al., 1996).

The UFL-AG-286 cell line has been shown to be highly susceptible and permissive to AgMNPV infection (Castro et al., 1997; Lynn, 2003; Sieburth and Maruniak, 1988b). A cellular sub-population of the UFL-AG-286 cell line (saUFL-AG-286), capable of growing in agitated suspension cultures, has been isolated and characterized (Gioria et al., 2006). An important feature of these cells is that they are active producers of ammonia, a by-product of cellular metabolism which may affect viral replication. A new low-cost serum-free medium designed specifically for the saUFL-AG-286 cell line has been developed, but the optimum combination of the multiplicity of infection and initial cell density for the production of AgMNPV OBs in suspension cultures of this cell line in the new medium has not yet been established.

The aim of this study was to investigate the effects of the variation of the multiplicity of infection and initial cell density on the production of AgMNPV OBs in suspension cultures of the saUFL-AG-286 cell line in a serum-free medium. Two sets of experiments following a 2^2 full factorial design with centre point (the two conditions of infection at two extreme levels, in the four possible combinations of conditions and levels, plus a further combination with each condition set at the middle of its extreme levels) were carried out in order to compare the performance of cultures infected in two different ranges of multiplicities of infection and initial cell densities. The results demonstrated the importance of an adequate selection of infection conditions to obtain high viral yields. They could also have implications on the development of a large-scale process for producing AgMNPV OBs in saUFL-AG-286 cultures in serum-free medium.

2. Materials and methods

2.1. Cell line

The saUFL-AG-286 insect cell line (Gioria et al., 2006) is a subpopulation of the UFL-AG-286 cell line (Sieburth and Maruniak, 1988a), established from embryos of *A. gemmatalis* and selected for its ability to grow in agitated suspension cultures, mainly in the form of isolated cells.

2.2. Culture medium

The medium used in the experiments was developed specifically for the saUFL-AG-286 cell line, with the following composition, in $g\,L^{-1}$: L-alanine, 0.10; L-arginine, 0.05; L-aspartic acid, 0.03; L-asparagine, 0.24; biotin, 2×10^{-4}; calcium pantotenate, 2×10^{-3}; casein hydrolysate (NZ Case Plus), 3.00; choline chloride, 0.02; cyanocobalamin, 4×10^{-4}; L-cisteine, 2×10^{-3}; folic acid, 4×10^{-4}; fumaric acid, 2×10^{-3}; glucose, 7.0; L-glicine, 0.06; L-glutamic acid, 0.06; L-glutamine, 2.46; L-histidine.HCl, 0.34; L-isoleucine, 5×10^{-3}; i-inositol, 4×10^{-4}; α-ketoglutaric acid, 0.015; L-leucine, 0.02; L-lisine.HCl, 0.06; L (−) malic acid, 0.027; L-metionine, 0.16; PABA, 4×10^{-4}; nicotinic acid, 4×10^{-4}; L-phenylalanine, 0.01; L-proline, 0.03; pyridoxine chloride, 4×10^{-4}; riboflavin, 4×10^{-4}; L-serine, 0.28; thiamine chloride, 4×10^{-4}; L-threonine, 0.017; tryptose phosphate broth, 1.00; L-tyrosine, 0.016; L-tryptophane, 0.01; L-valine, 0.01; yeast extract, 3.00; $NaHCO_3$, 0.35; NaH_2PO_4, 0.88; KCl, 0.28; $CaCl_2{\cdot}2H_2O$, 1.30; $MgSO_4$, 1.37; $MgCl_2{\cdot}6H_2O$, 2.30; $H_2MoO_4{\cdot}4H_2O$, 2×10^{-7}; $CoCl_2{\cdot}6H_2O$, 5×10^{-7}; $CuCl_2$, 5×10^{-7}; $MnCl_2{\cdot}4H_2O$, 5×10^{-8}; $ZnCl_2$, 3×10^{-7}. This basic formulation was supplemented with $15\,mL\,L^{-1}$ of a lipid microemulsion that was prepared according to Maiorella et al., 1988, but cod liver oil was replaced by soybean oil (Sojola®, AGD SRL, General Deheza, Argentina). The remaining chemicals were supplied by Sigma–Aldrich Co.

2.3. Suspension cultures of the saUFL-AG-286 cell line

Suspension cultures were maintained at 27 °C in 125 mL cylindrical glass flasks (12 mL working volume) shaking at 100 rpm on an orbital shaker (Forma Scientific, USA), in the serum-free medium. Subcultures were grown every 5 days, starting from an initial cell density of 3×10^5 viable cells per mL. Samples from saUFL-AG-286 suspension cultures were taken daily in order to follow the evolution of both cell density and viability. Cell density was determined using a Neubauer haemocytometer. Viability was assayed by the Trypan Blue dye exclusion method. Each sample was processed by duplicate counting of total and viable cells. The ammonia concentration was measured using a commercial kit, based on the Berthelot reaction, where the ammonia, in the presence of phenol and sodium hypochlorite, produces indophenol blue which is quantified spectrophotometrically at 540 nm (Wiener Lab., Rosario, Argentina).

2.4. Virus and virus quantitation

The strain of AgMNPV was isolated originally in Oliveros, Provincia de Santa Fe, Argentina (Claus et al., 1993). The virus stock used for these experiments was prepared by infection of suspension cultures of saUFL-AG-286 cells (2×10^5 cells mL^{-1}) at a MOI of 0.1 $TCID_{50}$ $cell^{-1}$ using the second passage after an intermediate amplification of the virus in *A. gemmatalis* larvae. Samples obtained from infected suspension cultures were processed as described for non-infected cultures. Additional samples were taken from cellular pellets for OBs quantitation. After extraction of cellular pellets with SDS 1%, the number of OBs was determined with a Neubauer haemocytometer by taking the average after counting three separate aliquots.

2.5. Experimental design and data analysis

The influence of the conditions of infection on the production of occlusion bodies of AgMNPV in serum-free suspension cultures of the saUFL-AG-286 cell line was investigated by two 2^2 full factorial assays with centre point (FFA + CP). A 2^2 full factorial assay with centre point is an experiment whose design consists of two factors, each with two levels, in which all possible combinations of factors and levels are assayed in four runs, plus one additional experimental run with each factor set up at the middle of its range (Box et al., 1978). In this study, each FFA + CP was set up for two factors (initial cell density and multiplicity of infection), with two coded levels (−1 and +1) and a centre point (0), and was run to evaluate the individual effects of the MOI and ICD, as well as the interaction between them. The responses evaluated in every run were the maximum density of viable cells (MVCD), the yield of occlusion bodies (OBY) and the cell specific yield of occlusion bodies (OBSY). The latter was calculated as the ratio between OBY and MVCD. Each experiment was performed in duplicate. The size of both main and interaction coefficients was calculated by multiple linear regression, and the statistical significance of the regression coefficients was estimated with the Student's *t*-test. In order to estimate the adequacy of the regression models, the analysis of variance (ANOVA) and the Fischer's *F*-test were used. A level of $p < 0.05$ was considered statistically significant. Experimental design and statistical analysis were aided by the JMP v.4 software.