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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

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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

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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.

Table 1
Experimental design and responses observed in the assay designed to study the effects of the conditions of infection MOI and ICD on the MVCD, the OBY and the OBSY in cultures of the cell line saUFL -AG-286 infected synchronously with AgMNPV in a medium free of serum.

Trial	Factor				Responses[a]		
	MOI ($TCID_{50}$ $cell^{-1}$)		ICD (cell mL^{-1})		MVCD (cells mL^{-1})	AgMNPV OBY (OBs mL^{-1})	AgMNPV OBSY (OBs $cell^{-1}$)
1	1[b]	5	−1	2×10^5	$2.49 \times 10^5 \pm 7.78 \times 10^3$	$1.37 \times 10^8 \pm 1.34 \times 10^7$	549 ± 36
2	+1	15	+1	8×10^5	$8.27 \times 10^5 \pm 3.82 \times 10^4$	$3.28 \times 10^8 \pm 1.63 \times 10^7$	397 ± 38
3	−1	5	+1	8×10^5	$1.12 \times 10^6 \pm 7.78 \times 10^4$	$1.81 \times 10^8 \pm 9.89 \times 10^7$	160 ± 78
4	+1	15	−1	2×10^5	$2.25 \times 10^5 \pm 7.07 \times 10^2$	$1.19 \times 10^8 \pm 1.06 \times 10^7$	528 ± 50
5	0	10	0	5×10^5	$6.02 \times 10^5 \pm 8.98 \times 10^4$	$3.34 \times 10^8 \pm 7.78 \times 10^6$	562 ± 97

[a] Data represent the average ± SD of two independent experiments.
[b] Numbers in the first column are the coded values of the variables.

3. Results

3.1. Infections with high values of MOI (synchronous infections)

Baculovirus-infected insect cell cultures usually express their maximum capacity of viral production when the infection takes place during the early to intermediate phase of exponential growth. Therefore, in order to evaluate the potential of production of AgMNPV OBs in saUFL-AG-286 cell cultures in a serum-free medium and, at the same time, determine the influence of the initial cell density and the multiplicity of infection on the viral yield, a 2^2 FFA + CP was carried out within an ICDs range between 2 and 8×10^5 cells mL^{-1} and a MOIs range of 5–15 $TCID_{50}$ $cell^{-1}$. The range of MOI values was selected in order to reach a probability level of simultaneous cellular infection higher than 99% (synchronous infection). Each infection was performed twice. A set of saUFL-AG-286 cell suspension cultures was infected with AgMNPV stock at different multiplicities of infection and times of culture (to attain the desired initial cell densities), according to the experimental design presented in Table 1. This Table also shows the responses determined for each combination of infection conditions. The progression of the infected cultures was followed up to day 5 post-infection; the count of total and viable cells and occlusion bodies associated with the cells was determined daily, as well as the concentration of ammonia.

As shown in Fig. 1, the cultures infected with AgMNPV almost did not multiply after infection. A non-infected control continued to grow up to a maximum cell density of 2.6×10^6 cells mL^{-1} (not shown). Growth after infection was affected by the conditions of infection: it was null in cultures which were infected at the highest value of MOI and the lowest value of ICD, while cultures infected with the lowest value of multiplicity of infection and the highest value of the initial cell density grew to a maximum density of viable cells 26% higher than the ICD. As a consequence of the interference

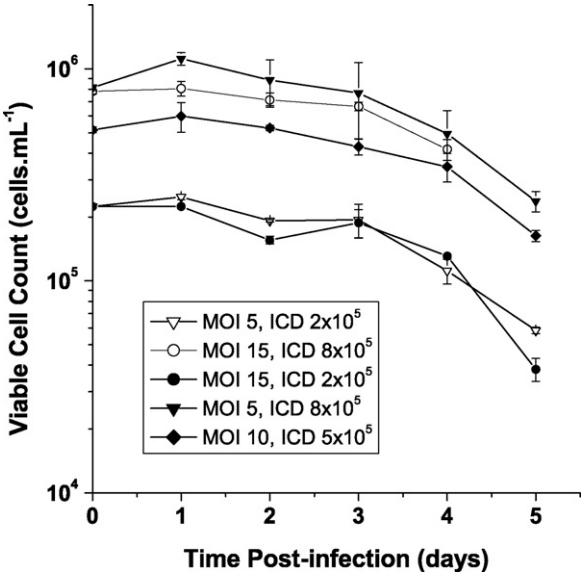

Fig. 1. Time course profiles of viable cells during AgMNPV infection of saUFL-AG-286 cell suspension cultures in serum-free medium. Cultures, grown in 100 mL shaker flasks with 12 mL working volume at 27 °C and 100 rpm were infected synchronously with different combinations of MOI and ICD, following the experimental design shown in Table 1.

with the cell multiplication after infection, the presence of three different patterns of progression in time can also be observed in Fig. 1. Each pattern corresponds to one of the three ICD values used in this design: the higher the initial cell density, the higher the value of the maximum density of viable cells. In turn, it can be observed that for each ICD, those cultures infected at the highest MOI tended to reach lower MVCD values. The statistical analysis of the regression coefficients, shown in Table 2A, allows to conclude

Table 2
Analysis of the main effects and interaction effects of the factors MOI and ICD over the responses MVCD (A), OBY (B) and OBSY (C) corresponding to the experiment in which cultures of the cell line saUFL-AG-286 were infected synchronously with AgMNPV in a medium free of serum.

Factor	Regression coefficient	Std. Error	*t*-value	*p*-value
(A)				
ICD (2×10^5, 8×10^5)	3.67×10^5	1.81×10^4	20.34	<0.0001*
MOI (5, 15)	-7.8×10^4	1.81×10^4	−4.32	0.0050*
ICD (2×10^5, 8×10^5) × MOI (5, 15)	-6.6×10^4	1.81×10^4	−3.66	0.0106*
(B)				
ICD (2×10^5, 8×10^5)	6.34×10^7	2.99×10^7	2.12	0.0785
MOI (5, 15)	3.21×10^7	2.99×10^7	1.07	0.3242
ICD (2×10^5, 8×10^5) × MOI (5, 15)	4.11×10^7	2.99×10^7	1.37	0.2184
(C)				
ICD (2×10^5, 8×10^5)	−127.38	34.90	−3.65	0.0107*
MOI (5, 15)	56.88	34.90	1.63	0.1543
ICD (2×10^5, 8×10^5) × MOI (5, 15)	67.38	34.90	1.93	0.1017

* Significant factors ($p < 0.05$).

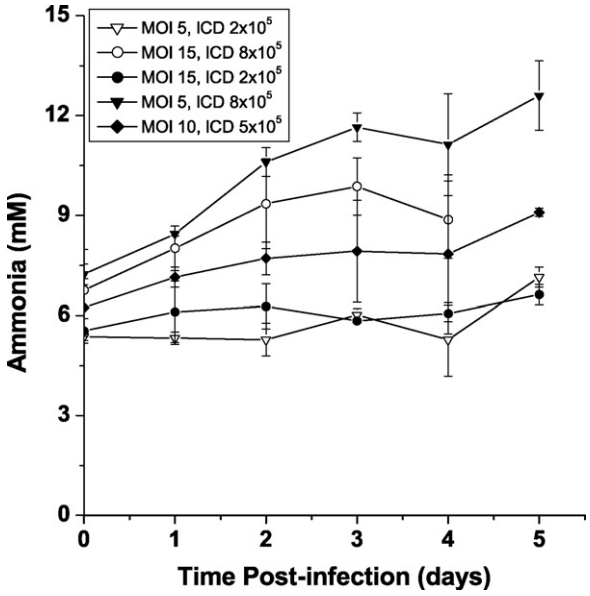

Fig. 2. Kinetics of ammonia accumulation in saUFL-AG-286 suspension cultures infected with AgMNPV in serum-free medium. Cultures, grown in 100 mL shaker flasks with 12 mL working volume at 27 °C and 100 rpm were infected with different combinations of MOI and ICD, following the experimental design shown in Table 1.

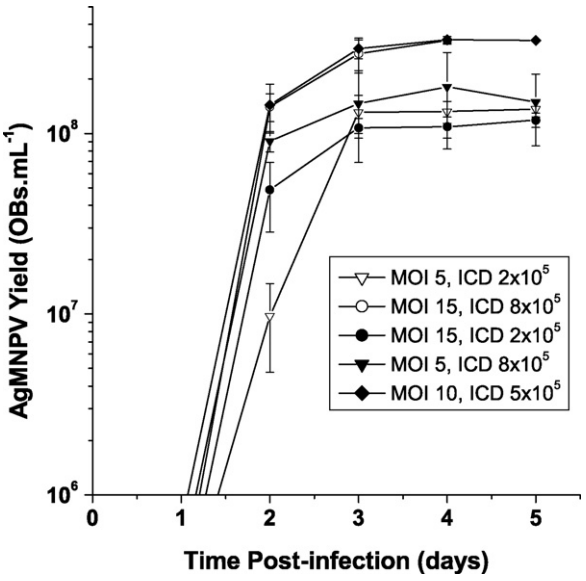

Fig. 3. Kinetics of production of OBs in saUFL-AG-286 suspension cultures infected with AgMNPV in serum free medium. Cultures, grown in 100 mL shaker flasks with 12 mL working volume at 27 °C and 100 rpm were infected with different combinations of MOI and ICD, following the experimental design shown in Table 1.

that the maximum density of viable cells was affected significantly by both the ICD and MOI, and that the interaction between both factors was also statistically significant.

The magnitude of the accumulation of ammonia in the supernatants of infected cultures was also affected by the conditions of infection, tending to be higher in those conditions of infection that allowed cellular multiplication, and lower in those cultures that did not grow after infection (Fig. 2). The ammonia concentration reached a maximum value of 12.60 mM in cultures infected with the lowest MOI and highest ICD, while the maximum concentration reached in cultures infected at the highest value of MOI and the lowest ICD was just 6.79 mM.

The progression of the OBs production as a function of post-infection time can be observed in Fig. 3. An OBs production peak was verified in every infected culture at day 2 post-infection. The initial OBs accumulation rate was more rapid in those cultures infected at higher ICD. The maximum OBs yields were reached between day 3 and day 5 post-infection, and an average maximum value of 3.34×10^8 OBs mL^{-1} was obtained in the cultures corresponding to the centre point of the experimental design, namely, those infected at a MOI of 10 $TCID_{50}$ cell^{-1} and an ICD of 5×10^5 cell mL^{-1}. The analysis of the regression coefficients showed that none of the factors under study had a statistically significant effect over the OBs yield (Table 2B). The lack of detection of significant effects of MOI and ICD on OBY, and the observation of a maximum OBY at the centre of the design, would indicate the inadequacy of first-order model to describe the influence of parameters of infection on the yield of occlusion bodies.

On the other hand, when the influence of the MOI and ICD on OBSY was analyzed it could be observed that the cells of the cultures infected at the lowest ICD produced an average of 538 OBs cell^{-1}, whereas the cells of the cultures infected at the highest ICD produced, on average, 279 OBs cell^{-1}. The analysis of the regression coefficients confirmed that the ICD exerted a significant influence on the specific production of OBs, while the variations in the MOI neither influenced significantly the OBSY nor was the existence of interactions between both factors demonstrated (Table 2C).

3.2. Infections with low values of MOI (asynchronous infections)

The previous experiment reveals the potential of production of AgMNPV occlusion bodies in saUFL-AG-286 cell cultures in the serum-free medium. However, the conditions employed in that experiment are not useful for establishing an industrial process since the infection at high MOI values demands a large amount of viral stock, one of the most expensive ingredients in the process of baculovirus production in cell cultures. Therefore, it was decided to evaluate the response of the system (saUFL-AG-286 – serum-free medium) to the AgMNPV infection at lower MOI values (asynchronous infection). Taking into account that, according to the results obtained in the previous experimental design,

Table 3
Experimental design and responses observed in the assay designed to study the effects of the conditions of infection MOI and ICD on the MVCD, the OBY and the OBSY in cultures of the cell line saUFL -AG-286 infected asynchronously with AgMNPV in a medium free of serum.

Trial	Factor				Responses[a]		
	MOI ($TCID_{50}$ cell^{-1})		ICD (cell mL^{-1})		MVCD (cell mL^{-1})	AgMNPV OBY (OBs mL^{-1})	AgMNPV OBSY (OBs cell^{-1})
1	−1[b]	0.1	−1	1.5×10^5	$4.45 \times 10^5 \pm 2.12 \times 10^4$	$2.86 \times 10^8 \pm 7.28 \times 10^7$	638 ± 133
2	+1	1	+1	4.5×10^5	$7.56 \times 10^5 \pm 2.12 \times 10^5$	$2.41 \times 10^8 \pm 3.82 \times 10^7$	339 ± 146
3	−1	0.1	+1	4.5×10^5	$1.26 \times 10^6 \pm 3.11 \times 10^5$	$6.17 \times 10^7 \pm 4.69 \times 10^7$	55 ± 51
4	+1	1	−1	1.5×10^5	$2.60 \times 10^5 \pm 1.27 \times 10^4$	$1.79 \times 10^8 \pm 1.41 \times 10^7$	690 ± 88
5	0	0.55	0	3×10^5	$5.60 \times 10^5 \pm 1.99 \times 10^5$	$2.94 \times 10^8 \pm 7.85 \times 10^7$	532 ± 50

[a] Data represent the average ± SD of two independent experiments.
[b] Numbers in the first column are the coded values of the variables.

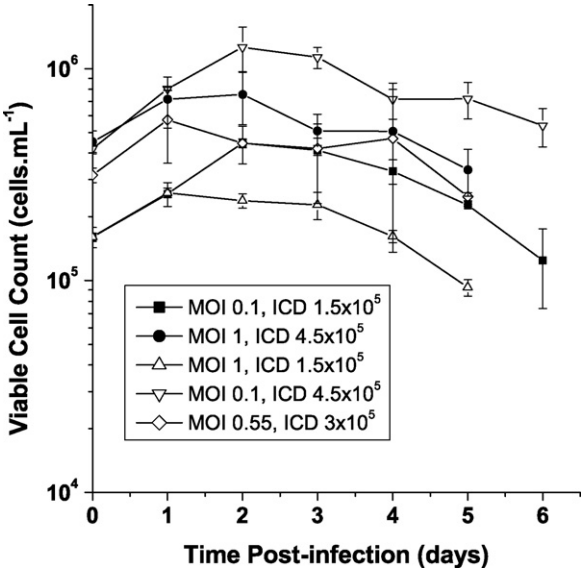

Fig. 4. Time course profiles of viable cells during AgMNPV infection of saUFL-AG-286 cell suspension cultures in serum-free medium. Cultures, grown in 100 mL shaker flasks with 12 mL working volume at 27 °C and 100 rpm were infected with different combinations of MOI and ICD, following the experimental design shown in Table 3.

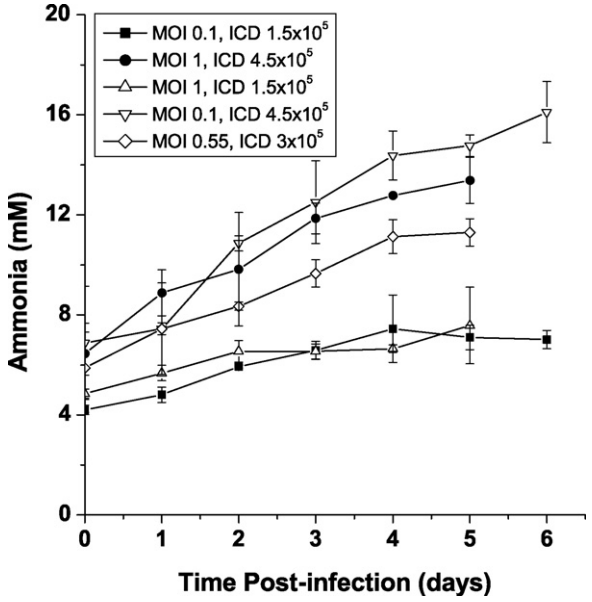

Fig. 5. Kinetics of ammonia accumulation in the supernatants of saUFL-AG-286 suspension cultures infected with AgMNPV in serum-free medium. Cultures in 100 mL shaker flasks with 12 mL working volume at 27 °C and 100 rpm were infected with different combinations of MOI and ICD, following the experimental design shown in Table 3.

the saUFL-AG-286 cells in the serum-free medium reduced their specific capacity of OBs production as the ICD increased, and considering that in the cultures infected at low MOI values a higher post-infection proliferation level is expected, a range of ICDs lower than in the previous experiment was selected for the new experimental design. A 2^2 FFA + CP was performed, within an ICDs range between 1.5 and 4.5×10^5 cell mL^{-1}, and a MOIs range between 0.1 and 1 $TCID_{50}$ $cell^{-1}$, according to the design presented in Table 3, in which the experimental responses (MVCD, OBY and OBSY) are also shown.

Fig. 4 shows that all cultures multiplied after the infection but no common progression patterns of viable cells counts can be observed. As expected, the percentage of growth after infection was higher than that observed in the previous experiment, varying between 40% and 206% with respect to the respective ICDs. The highest levels of cellular multiplication were reached in cultures infected at the lowest MOI, with an average of 174%, while in the cultures infected at the highest value of the multiplicity of infection the average proliferation was 71%. The maximum viable cell density was affected by the variations of both the initial cell density and the multiplicity of infection. While the ICD increase determined the MVCD increase, the MOI effect was the opposite. The statistical analysis showed that both parameters exerted a significant effect over MVCD, and no significant interaction between both factors was obtained (Table 4A).

The increased level of cellular multiplication achieved in these cultures was accompanied by an increased level of accumulation of ammonia in the supernatants of infected cultures. (Fig. 5). The quantity of ammonia accumulated was related to the initial cell density: the cultures which were infected at the highest value of the ICD tended to accumulate higher concentrations of ammonia (average 14.74 mM) than those that were infected at the lowest value (average 7.70 mM).

The initial accumulation rate of occlusion bodies in these experiments was lower than in the cultures infected at high MOI, especially in those cultures infected at MOI 0.1 (Fig. 6). Maximum yields were reached between days 5 and 6 post-infection. The average values of the yield of occlusion bodies varied between 6.17×10^7 OBs mL^{-1}, in cultures infected at MOI 0.1 $TCID_{50}$ $cell^{-1}$ and ICD 4.5×10^5 cell mL^{-1}, and 2.94×10^8 OBs mL^{-1}, in cultures corresponding to the centre point of the design (MOI 0.55 $TCID_{50}$ $cell^{-1}$, ICD 3×10^5 cell mL^{-1}). As it was observed in the

Table 4
Analysis of the main effects and interaction effects of the factors MOI and ICD over the responses MVCD (A), OBY (B) and OBSY (C) corresponding to the experiment in which cultures of the cell line saUFL-AG-286 were infected asynchronously with AgMNPV in a medium free of serum.

Factor	Regression Coefficient	Std. Error	*t*-value	*p*-value
(A)				
ICD (1.5×10^5, 4.5×10^5)	3.37×10^5	7.26×10^4	5.12	0.0022*
MOI (0.1, 1)	-1.91×10^5	6.57×10^4	−2.64	0.0387*
ICD (1.5×10^5, 4.5×10^5) × MOI (0.1, 1)	-8.86×10^4	7.26×10^4	−1.22	0.2680
(B)				
ICD (1.5×10^5, 4.5×10^5)	-4.84×10^7	2.86×10^7	−1.87	0.1112
MOI (0.1, 1)	2.02×10^7	2.59×10^7	0.71	0.5062
ICD (1.5×10^5, 4.5×10^5) × MOI (0.1, 1)	7.94×10^7	2.86×10^7	2.77	0.0323*
(C)				
ICD (1.5×10^5, 4.5×10^5)	−240.19	41.95	−6.32	0.0007*
MOI (0.1, 1)	93.33	38.00	2.22	0.0678
ICD (1.5×10^5, 4.5×10^5) × MOI (0.1, 1)	64.44	41.95	1.54	0.1754

* Significant factors ($p < 0.05$).

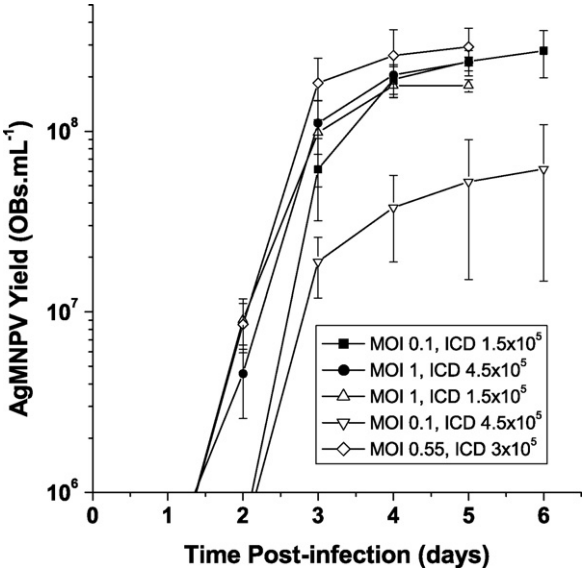

Fig. 6. Kinetics of production of OBs in saUFL-AG-286 suspension cultures infected with AgMNPV in serum-free medium. Cultures in 100 mL shaker flasks with 12 mL working volume at 27 °C and 100 rpm were infected with different combinations of MOI and ICD, following the experimental design shown in Table 3.

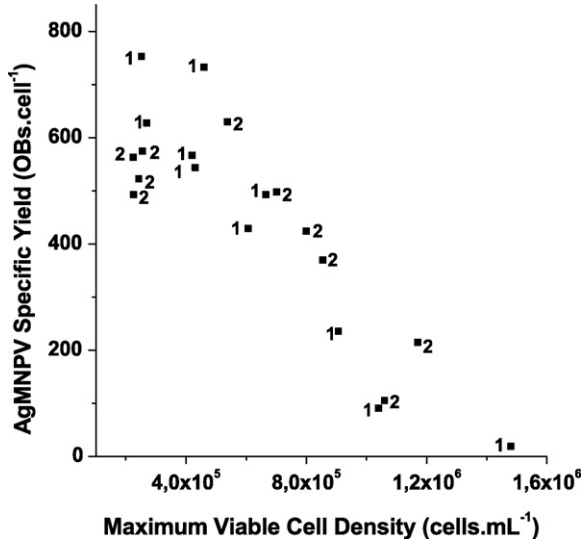

Fig. 7. Plot of OBSY against the MVCD attained in saUFL-AG-286 cell suspension cultures infected with AgMNPV with different combinations of MOI and ICD, in a serum-free medium. (1) Data corresponding to the experiment in which cultures were infected synchronously; (2) data corresponding to the experiment in which cultures were infected asynchronously.

first FFA + CP, the analysis showed that none of the conditions under study had a statistically significant effect over the yield of occlusion bodies, although a significant interaction between both conditions could be revealed (Table 4B). The cellular specific yield of occlusion bodies also decreased in these experiments with the ICD increases: while the cells of cultures infected at an ICD of 1.5×10^5 cell mL^{-1} produced on average 664 OBs cell^{-1}, those infected in cultures with an ICD of 4.5×10^5 cell mL^{-1} produced on average 197 OBs cell^{-1}. On the other hand, the MOI increase was associated with a slight increment in the cellular specific yield of OBs. The analysis showed that ICD exerted a significant effect on OBSY, while the influence of MOI did not reach statistical significance (Table 4C).

3.3. Modelling and statistical optimization of AgMNPV production

Given the similarity of behaviour of MVCD with respect to the influence of the conditions of infection in cultures infected either synchronously (high MOI) or asynchronously (low MOI), the possibility of establishing a single model to represent the influence of the MOI and the ICD on the MVCD was assessed through multiple linear regression of all data obtained in both FFA + CP. A significant first-order model was obtained (Table 5), which gave the following equation that makes it possible to estimate the value of MVCD as a function of the values assigned to the MOI and ICD.

$$\text{MVCD} = 2.04 \times 10^5 - 2.25 \times 10^4 \times \text{MOI} + 1.27 \times \text{ICD} \quad (1)$$

Table 5
ANOVA of the model applied for MVCD[a] as a function of the MOI and the ICD according to the full set of data obtained in the experiments in which cultures of saUFL-AG-286 cells were infected either synchronously or asynchronously with AgMNPV in a medium free of serum.

Source of variation	DF[b]	Sum of squares	Mean square	*F* value	*p* value
Model	2	1.53×10^{12}	7.67×10^{11}	49.114	<0.0001
Residual	17	2.50×10^{11}	1.56×10^{10}		
Lack of fit	7	1.49×10^{11}	2.13×10^{10}	1.904	0.1815
Pure error	10	1.01×10^{11}	1.12×10^{10}		
Total	19	1.78×10^{12}			

[a] $r^2 = 0.86$; adjusted $r^2 = 0.84$.
[b] Degrees of freedom.

In turn, MVCD could constitute a unifying element to represent also the variation of OBSY and OBY. Confirming this presumption, when the cellular specific yield of occlusion bodies is represented against the maximum density of viable cells, the response patterns obtained in both factorial assays are almost coincident (Fig. 7). This figure allows us to observe that the cellular capacity to produce OBs was reduced linearly with the increase of MVCD.

On the other hand, when the yield of occlusion bodies is represented as a function of the maximum density of viable cells (Fig. 8), it can be noticed that the patterns obtained in both factorial assays are also almost coincident. The highest OB yields tended to concentrate in those cultures whose MVCD values were between 4 and 8×10^5 viable cells mL^{-1}. An average yield of 3.05×10^8 OBs mL^{-1} was reached in those cultures whereas in cultures with MVCD val-

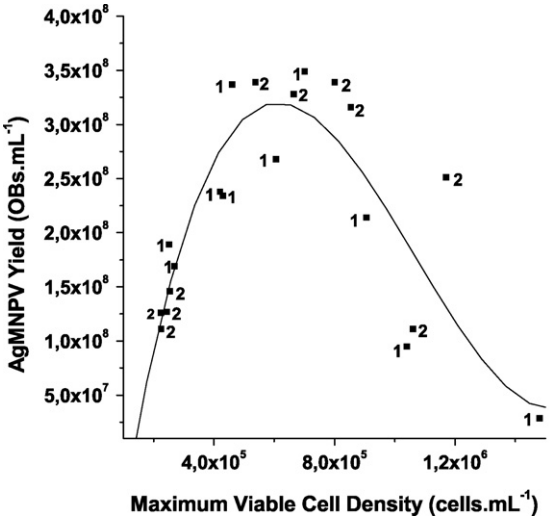

Fig. 8. Plot of OBY against the MVCD reached in saUFL-AG-286 cell suspension cultures infected with AgMNPV with different combinations of MOI and ICD, in a serum-free medium. (1) Data corresponding to the experiment in which cultures were infected synchronously; (2) data corresponding to the experiment in which cultures were infected asynchronously. The line represents the model obtained by adjusting the data using a third-order polynomial function.

Table 6
ANOVA of the model applied for OBY[a] as a function of MVCD according to the full set of data obtained in the experiments in which cultures of saUFL-AG-286 cells were infected either synchronously or asynchronously with AgMNPV in a medium free of serum.

Source of variation	DF[b]	Sum of squares	Mean square	*F* ratio	*p* value
Model	3	1.36×10^{17}	4.52×10^{16}	15.287	<0.0001
Residual	16	4.74×10^{16}	2.96×10^{15}		
Total	19	1.83×10^{17}			

[a] $r^2 = 0.74$; adjusted $r^2 = 0.69$.
[b] Degrees of freedom.

ues which were lower or higher than the ones within the range referred to above, an average yield of $1{,}43 \times 10^8$ OBs mL^{-1} was reached. The relation between the OBY and the MVCD can be adjusted through a polynomial model of third-order, which makes it possible to estimate the value of OBY through the following regression equation:

$$\mathrm{OBY} = -2.42 \times 10^8 + 2.12 \times 10^3\,\mathrm{MVCD} - 2.42 \times 10^{-3}\,\mathrm{MVCD}^2 + 7.57 \times 10^{-10}\mathrm{MVCD}^3 \quad (2)$$

Fischer's test yielded a high significance for the regression (Table 6).

From Eqs. (1) and (2), Eq. (3) is obtained which allows estimating the yield of AgMNPV OBs from the values assigned to the MOI and ICD:

$$\begin{aligned}\mathrm{OBY} = {} & 9.54 \times 10^7 - 2.76 \times 10^7 \times \mathrm{MOI} + 1.56 \times 10^3 \times \mathrm{ICD} \\ & - 1.11 \times 10^2 \times \mathrm{MOI} \times \mathrm{ICD} - 9.85 \times 10^5 \times \mathrm{MOI}^2 \\ & - 3.15 \times 10^{-3} \times \mathrm{ICD}^2 + 1.46 \times \mathrm{MOI}^2 \times \mathrm{ICD} \\ & - 8.25 \times 10^{-5} \times \mathrm{MOI} \times \mathrm{ICD}^2 - 8.63 \times 10^3 \times \mathrm{MOI}^3 \\ & + 1.54 \times 10^{-9} \times \mathrm{ICD}^3 \end{aligned} \quad (3)$$

In order to validate the model and, at the same time, verify the reproducibility of the yields of OBs in different batches of the serum-free medium, infections were carried out in a new batch of medium, using two different combinations of MOI and ICD, as shown in Table 7. The table also shows the yields of AgMPNV OBs obtained and the values estimated according to the model. Each experiment was performed in duplicate. As can be observed, the yields reached under both experimental conditions are almost similar to those estimated according to the model. The results of these validation experiments demonstrate the usefulness of the model to establish a combination of infection parameters that allows maximizing the yield of occlusion bodies of AgMNPV in cultures of infected saUFL-AG-286 cells in different batches of a new culture medium free of serum.

4. Discussion

The results of this study demonstrate that suspension cultures of the cell line saUFL-AG-286 in a new culture medium free of serum can produce high yields of occlusion bodies of the *Anticarsia gemmatalis multiple nucleopolyhedrovirus*. Yields of occlusion bodies obtained in most experiments in this study, carried out under different conditions of infection, were higher than those obtained previously for the same virus. Claus et al. 1993, in static cultures of the IPLB-Sf-21 cell line, obtained yields of up to 3.15×10^7 OBs mL^{-1} employing the TC-100 culture medium, containing serum or an egg-yolk emulsion. Visnovsky and Claus, 1994, in suspension cultures of the same line in a serum-containing medium reached a maximum yield of 2×10^7 OBs mL^{-1}. Later, Grasela and McIntosh, 1998 assayed the replication of AgMNPV in static cultures of seven different cell lines, in different serum-containing media, producing OBs in only two of them with a maximum yield of 7.5×10^6 OBs mL^{-1} in the BCIRL-HV-AM1 line. Batista et al., 2005, in suspension cultures of the Sf9 line in different culture media, obtained yields of up to 1.80×10^7 OBs mL^{-1}. Rodas et al., 2005, on the other hand, reached maximum yields of about 2×10^8 OBs mL^{-1} in suspension cultures of the Sf9 line in the Sf900 II commercial serum-free medium. Gioria et al., 2006, employing suspension cultures of the saUFL-AG-286 line, but in a TC-100 serum-containing culture medium, reached a maximum yield of 5.80×10^7 OBs mL^{-1}. The maximum yield of OBs reached in the current study was, therefore, at least 60% higher than the highest yield previously obtained for the same virus in cell cultures. In addition, the yield of AgMNPV OBs reached in this study is comparable to those obtained during the production of occlusion bodies of other baculoviruses in insect cell cultures (Chakraborty et al., 1999; McKenna et al., 1997; Lua and Reid, 2000; Lua and Reid, 2003; Pedrini et al., 2006).

The high yields achieved in most of the infection conditions which were tested during this study are the product of the very high individual capacity of infected cells to produce occlusion bodies. In 90% of the experiments performed in this work, the specific yield of OBS was over 100 OBs cell^{-1}, with a maximum value greater than 700 OBs cell^{-1}. This high specific capacity to produce OBs of AgMNPV seems to be a characteristic property of the saUFL-AG-286 cell line in what concerns to the replication of this virus, as in previous published papers, which were performed in suspension cultures of other cell lines, the specific yields of occlusion bodies were lower than those achieved in these experiments (Batista et al., 2005; Rodas et al., 2005; Visnovsky and Claus, 1994). The high capacity of the cell line UFL-AG-286, predecessor of the cell line saUFL-AG-286, to replicate AgMNPV has been documented previously (Castro et al., 1997; Lynn, 2003). Another factor to be taken into account to explain the high specific yields of OBs obtained in this study is the culture medium. The infection with AgMNPV of the same saUFL-AG-286 cell line in the serum-containing TC-100 medium allowed us to reach a specific yield of 64 OBs per cell (Gioria et al., 2006), less than half the average specific yield achieved in this work under similar conditions of infection. The high production of OBs in the serum-free medium specifically designed for saUFL-AG-286 cells, in comparison with the yield reached in the TC-100 medium supplemented with serum, is the result of its nutrient composition. The strategy to design and optimize the composition of the new medium was governed by two main criteria: the ability to promote the growth of saUFL-AG-286 cell cultures and, on the other hand, the capability to improve the quantitative yield of biologically active AgMNPV OBs (Gioria et al., unpublished results). Summing up, it is concluded that the large specific yield of occlusion bodies

Table 7
Validation of the model. Two different combinations of the ICD and the MOI were assayed, each one of them in duplicate, in cultures of saUFL-AG-286 cells infected with AgMNPV in a new batch of the serum-free medium.

Experiment	ICD (cells mL^{-1})	MOI ($TCID_{50}$ cell^{-1})	OBY[a] (OBs mL^{-1})	OBY predicted[b] (OBs mL^{-1})
1	4.50×10^5	10	$2.89 \times 10^8 \pm 4.17 \times 10^7$	3.23×10^8
2	2.50×10^5	0.5	$3.20 \times 10^8 \pm 1.70 \times 10^7$	3.20×10^8

[a] Data represent the average ± standard deviation of two independent experiments.
[b] Calculated according to Eq. (3).

of the AgMNPV virus in saUFL-AG-286 cell line cultures would be a consequence of both the intrinsic ability of the cells to replicate a virus to which they are naturally susceptible and permissive, and of a culture medium which is apt to sustain viral replication and production of occlusion bodies.

The statistical analysis shows that the OBs specific yield was influenced significantly only by the ICD, and that it was not affected by the MOI variations (Tables 2C and 4C). The ICD increases were associated with a reduction in the OBs specific yields. The ICD effect upon the specific yield can be explained by its significant influence upon MVCD (Tables 2A and 4A), and also by considering the linear relationship between the MVCD increments and the reduction of the OBs specific yields that can be observed in Fig. 5a. This phenomenon is similar to the well-known "cell density effect", which limits virus replication (Wood et al., 1982) and the production of recombinant proteins (Taticek and Shuler, 1997; Wong et al., 1996; Yang et al., 1996) in the baculovirus-insect cell system when the cell cultures are infected at high cell density. Several reasons, including nutrient depletion (Bédard et al., 1994; Elias et al., 2000), toxic waste products accumulation (Taticek and Shuler, 1997), autocrine factors and cell cycle distribution (Braunagel et al., 1998; Doverskog et al., 2000; Calles et al., 2006), have been proposed to explain the "cell density effect", but the causes remain to be identified. A nutritional limitation does not seem to be a plausible explanation for the continuing decline in the specific yield of occlusion bodies of AgMNPV with the increase in the maximum cell density of saUFL-AG-286 cell cultures if one considers that the effect is intensified continuously from very low cell densities as to be caused by the running out of some nutrient required for viral replication or OBs production. An alternative explanation is that this effect could be caused by the accumulation of metabolic products toxic for animal cells, such as lactate and ammonia, which could interfere with the viral replication. The saUFL-AG-286 cell cultures do not accumulate lactate but, instead, excrete ammonia (Gioria et al., 2006). The latter is an inhibitor of the baculovirus replication and the expression of recombinant proteins (Taticek and Shuler, 1997). Fig. 9 shows how the increased concentration of ammonia in cultured cells is correlated with the reduction of the specific yield of occlusion bodies. This fact constitutes a strong argument in favour of the role of ammonia as an inhibitor of the production of occlusion bodies of AgMNPV in saUFL-AG-286 cell cultures. However, additional experiments will be needed to determine whether the reduced cellular capacity to produce OBS is a consequence of the increase in the concentration of ammonia, or whether both facts are independent consequences of the increase of the cellular density.

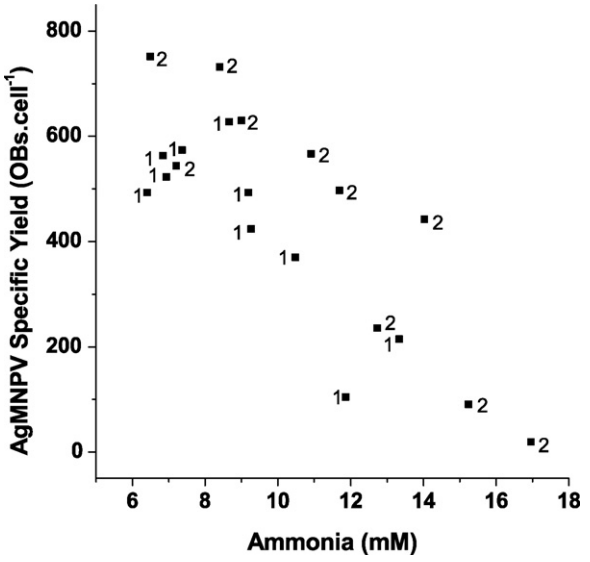

Fig. 9. Plot of OBSY against the concentration of ammonia accumulated in the supernatants of saUFL-AG-286 cell suspension cultures infected with AgMNPV with different combinations of MOI and ICD, in a serum-free medium.

Despite the continuous reduction of the specific yield of AgMNPV OBs associated with the increase of the maximum cell density in saUFL-AG-286 cell cultures, the volumetric yield decayed only in cultures with a maximum cell density over 8×10^5 cells mL^{-1}, and even increased with the increase of the MVCD when it ranged between 2.5 and 4.0×10^5 cell mL^{-1}, as shown in Fig. 8. This pattern can be explained by the balance between the increase in the number of OBs producing cells and the reduction of their individual production capacity as the cell density increases. When the maximum cell density reaches values between 4 and 8×10^5 cells mL^{-1}, the increase in the number of producing cells equilibrates the reduction of the specific yield, and the volumetric yield remains almost constant in its maximum value. In order to reach a maximum volumetric yield of OBs, it is then necessary to select infection conditions which allow the saUFL-AG-286 cell culture infected with AgMNPV to reach a value of its maximum cell density between that range of values, since higher or lower maximum cell densities would determine a sub-optimal volumetric yield.

By means of the relationship found between the volumetric yield and the maximum viable cell density, on the one hand, and between the latter and the MOI and the ICD on the other, it was possible to obtain an equation linking the volumetric yield with the infection parameters. It is interesting to remark that the use of the MVCD introduces a unifying element for the analysis of the behaviour of this baculovirus infection system that is independent of the working range of infection conditions. Therefore, it becomes useful for predicting the OBs yield in a production process under any combination of infection parameters. Even though the concept of introducing the MVCD as a unifying element could be generalized to the statistical modelling of any system of either OBs or recombinant proteins production in insect cell cultures (Wong et al., 1996), the specific model developed in this paper can only be applied to the production of AgMNPV OBs in suspension cultures of saUFL-AG-286 cells in the new serum-free medium employed in this study.

An important consequence derived from these results and reflected in the model is that different combinations of the ICD and MOI infection parameters can determine the same maximum density of viable cells and, consequently, the same volumetric yield of occlusion bodies. Any combination of these parameters which allows reaching a maximum cell density within the 4 and 8×10^5 cells mL^{-1} range will, in turn, obtain a maximum OBs yield, demonstrated in the experiments that validated this model. Another important consequence of this fact is that it is possible to reach maximum OBs yields by infecting the cultures early and at low multiplicity of infection. Infecting cultures under these conditions minimizes the viral stock consumption necessary to start the infection. It has been demonstrated by this study that it is possible to save up to 97% viral stock by selecting the adequate combination of the MOI and the ICD, without impairing the final volumetric yield of OBs. In a large-scale production process of an insecticide baculovirus, the preparation of the amount of budded virus necessary to infect the production reactor requires a sequence of viral amplification of successive steps, from the seed stock up to the inoculation reactor. This process parallels the amplification of the cell culture, with a significant impact on the global economy of the process (Rhodes, 1996). Besides, the genetic stability of the virus can be compromised through the successive amplification rounds, finally affecting both the process yield and the quality of the product (Krell, 1996; Lua et al., 2002; Soares de Rezende et al., 2009). Therefore, the reduction of the amount of virus required to start the infection process impacts positively upon

both the economic feasibility of the process and the quality of the product.

In conclusion, the current study has demonstrated that the infection of suspension cultures of the saUFL-AG-286 cell line with *Anticarsia gemmatalis multiple nucleopolyhedrovirus* in a serum-free, low-cost medium allows to obtain yields of occlusion bodies higher than 3×10^8 OBs mL^{-1}. The analysis of the influence of infection parameters allowed us to demonstrate that both the cell specific capacity of OBs production and the volumetric yield mainly depend on the maximum density of viable cells. The latter, in turn, depends on the selection of the initial cell density and multiplicity of infection. From the regression analysis of the obtained results, an empirical model was derived which proved to be useful in establishing the combination of infection parameters that allows a maximum yield of occlusion bodies with the least viral stock consumption. These results could contribute to the development of technically and economically feasible processes to produce viral insecticides in lepidopteran insect cell cultures.

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