Adaptation of the Se301 insect cell line to suspension culture. Effect of turbulence on growth and on production of nucleopolyhedrovirus (SeMNPV)

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Abstract As chemical pesticides are being banned as control agents for agricultural pests, the use of the highly specific, safe to non-target organisms baculoviruses has been proposed. These viruses can be produced either in vivo or in vitro. In vitro production requires appropriated host insect cell lines with the ability for growing as freely-suspended cells. In this work, the *Spodoptera exigua* Se301 cell line was used to produce the commercially available *S. exigua* nucleopolyhedrovirus (SeMNPV) in suspension. Se301 cells showed to be very sensitive to the hydrodynamic shear rates developed in bioreactors. A process of progressive adaptation to freely-suspended cultures using protective additives against shear stress and disaggregant was proposed. The best combinations were polyvinyl alcohol (PVA) or polyvinyl pyrrolidone (PVP) with the disaggregant dextran sulfate (DS). Both static and freely-suspended Se301 cell cultures were successfully infected with the SeMNPV baculovirus. Production of occluded baculovirus (OB) increased with the multiplicity of infection (MOI > 0.1).

Keywords Shear rate · Insect cell · Baculovirus · Damage · *Spodoptera* · Turbulence

Introduction

Spodoptera exigua is a major pest of worldwide importance, especially in covered crops (Murillo et al. 2006a). In the greenhouses of southeastern Spain (Almería, Spain) *S. exigua* has developed resistance to many chemical pesticides (Muñoz et al. 1997). Baculoviruses are well known for their potential as agents for biological control of pests in agriculture and forestry (Kolodny-Hirsch et al. 1993; Szweczyk et al. 2006). They do not harm non-target organisms and have a high specificity (Szweczyk et al. 2006). The use of the highly pathogenic and specific nucleopolyhedrovirus of *S. exigua* (SeMNPV) was proposed to fight against larvae of *S. exigua* (Kolodny-Hirsch et al. 1993; Smits and Vlaskov 1988). This virus is commercially available in Spain, the United States, Thailand and The Netherlands, under the name Spod-XTM (Moscardi 1999). Nowadays, baculoviruses as pest control agents are mainly produced in vivo.

The baculovirus-insect cell system is considered as a powerful biotechnological tool to produce various valuable biotechnological products (Maranga et al. 2004; Van Lier et al. 1996; Cruz et al. 1998; Negrete and Kotin 2007; Szweczyk et al. 2006). However, considering that the technology for the in vitro protein expression is well-established (Agathos 1996), the in vitro production of baculovirus is an attractive cost-effective alternative (Enden et al. 2005). Although some insect host cell lines have

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been adapted to be cultured in bioreactors as freely-suspended cells (Possee et al. 1997), the adaptation generally presents important obstacles, especially in large-scale culture systems. For example, cell damage associated with shear rates developed in bioreactors is frequently observed (Wu et al. 1990).

Many insect cell lines are anchorage-dependent for growth (Papoutsakis 1991a) and tend to form clumps. Therefore, for any bioprocess it is necessary to previously adapt them to grow in suspension. This can be achieved with continuous exposure to progressively higher agitation intensities to obtain a single cell population (Papoutsakis 1991a) and, if needed, the use of disaggregant agents. Taticek et al. (1997) used heparin with relative success in agitated cultures of *T. ni* (Tn5B1-4): after three passages the cells continued to present a healthy growth. Using compounds with a similar structure to heparin, Dee et al. (1997a) found that dextran sulfate was the most effective in the dispersion of Tn5B1-4 clumps. They needed concentrations of $25 \mu\text{g mL}^{-1}$ for cells cultured in serum-free media, and up to $300 \mu\text{g mL}^{-1}$ for cells cultured in serum containing media. It was also observed that the higher the degree of sulfation, the lesser the degree of aggregation, concluding that the reduction of cell clumping is an ion-specific effect.

To mitigate the harmful effects of certain shear stress levels on insect cells, different additives with protective effects have been proposed (Chattopadhyay et al. 1995; Chisti 2000). The protection mechanisms and their effect depend greatly on the additive properties, the amount added and the cell line. Depending on whether or not the additives alter the rheological properties of the medium (García Camacho et al. 2001; Michaels and Papoutsakis 1991), different types of protective actions (i.e. physical or biological, or both of them) have been attributed to these compounds. Satisfactory results in the adaptation to suspension culture have been reported for several insect cell lines (e.g. *T. ni* or *S. frugiperda*) in which different additives were employed, such as serum, Pluronic F68 (PF68), methylcellulose, polyvinyl alcohol (PVA), polyethylene glycol (PEG), polyvinyl pyrrolidone (PVP), and dextrans (Van Lier et al. 1996; Wu et al. 1990; Papoutsakis 1991b; Palomares et al. 2000; Chattopadhyay et al. 1995; Dee et al. 1997a, b).

In this work, it has been studied several of the commercially available protective additives in the adaptation to suspension culture of the cell line Se301

of *S. exigua*. The influence of the agitation intensity and the multiplicity of infection (MOI) on the production of an autochthonous occluded baculovirus (Murillo et al. 2006b), marketed by the Spanish company Biocolor S.L. under the name of Vir-ex[®], was also studied.

Materials and methods

Culture media, additives, cell line and baculovirus

The *S. exigua* Se301 cell line, kindly donated by the Department of Virology of Wageningen University, was used in all experiments. In the whole experimentation, the insect cell TNM-FH medium (Sigma-Aldrich, Ref. T3285) was used, supplemented with a 10% of fetal bovine serum (FBS, Sigma-Aldrich, Ref. F7524), 10 mL L^{-1} of a penicillin–streptomycin solution (Sigma-Aldrich, Ref. P4333) and $500 \mu\text{L L}^{-1}$ of an amphotericin B solution (Sigma-Aldrich, Ref. A2942). Attached cells were maintained in 25 cm^2 T-flasks (Sigma-Aldrich, Ref. F7177) at 27°C in an incubator.

In suspension cultures, different protective additives against shear stress were evaluated: PF68 (Sigma-Aldrich, Ref. P1300, average molecular weight 8,350), low viscosity Carboxymethylcellulose (CMC, Sigma-Aldrich, Ref. C5678), PEG (Sigma-Aldrich, Ref. 95904, molecular weight 3,500–4,500), PVA (Sigma-Aldrich, Ref. P8136, average molecular weight 30,000–70,000), and PVP (Sigma-Aldrich, Ref. P2307, average molecular weight 10,000). Anticoagulant additives were also evaluated: heparin (Sigma-Aldrich, Ref. H4784) and dextran sulfate (DS, Sigma-Aldrich, Ref. 31,404, average molecular weight 5,000).

The budded form of the autochthonous virus of SeMNPV was extracted in the haemolymph of infected larvae, according to the modified protocol from Zhang et al. (2006). Third and fourth instar larvae of *S. exigua* infected *per os* with the occluded form of the virus were provided by Biocolor, S.L.

Studies of potential toxicity of protective and anticoagulant additives

Additives have the potential to be toxic to a microorganism either directly or indirectly. Therefore, the

possible effects of the aforementioned additives on the growth of Se301 were assessed in static cultures at various concentrations.

All assays were carried out in 12.5 cm² T-flasks with 2 mL of fresh TNMFH medium supplemented with each additive at the concentrations shown in Table 1 and at an initial cell density of 2.5×10^5 cell mL⁻¹. There were also static control cultures but

without additive. All experiments, including the controls, were performed in duplicate and maintained for 5 days at 27 °C in an incubator. Cells were detached daily for concentration and viability determinations.

Total cell concentration was determined using a hemocytometer (Neubauer chamber) in a light microscope. Viable cell concentration was determined with

Table 1 Additives and concentrations used in the evaluation of their toxicity on the Se301 cell line

	Concentration of additive (% w/v)										
	0.01	0.025	0.05	0.1	0.15	0.2	0.3	0.4	0.6	0.8	1
PF68											
$C_f \times 10^{-5}$	6.3		6.3	6.1	5.0	4.0	2.6	0.6			
V	91		92	88	83	75	74	42			
μ_{app}	0.298		0.297	0.290	0.260	0.200	0.150	0.033			
k_d	0.004		0.005	0.012	0.042	0.102	0.152	0.269			
k_{df}	0.001		0.000	0.003	0.002	0.002	0.002	0.002			
CMC											
$C_f \times 10^{-5}$		6.3	6.4	6.2		6.5		6.2	6.4	5.5	5.5
V		89	91	90		90		88	89	90	89
μ_{app}		0.300	0.302	0.299		0.307		0.296	0.302	0.299	0.295
k_d		0.002	0.000	0.003		-0.005		0.006	0.000	0.003	0.007
k_{df}		0.0011	0.001	0.002		-0.001		0.002	0.001	0.100	0.135
PVA											
$C_f \times 10^{-5}$		6.7	6.8	6.5		6.7		6.3	6.2	5.4	5.3
V		91	90	88		90		91	91	90	88
μ_{app}		0.300	0.310	0.300		0.316		0.296	0.292	0.298	0.234
k_d		0.002	-0.009	0.002		-0.014		0.005	0.009	0.004	0.067
k_{df}		-0.0018	-0.002	-0.002		-0.002		0.001	0.001	0.065	0.123
PVP											
$C_f \times 10^{-5}$		6.5	6.4	6.5		6.6		6.2	6.1	5.2	5.1
V		90	88	89		88		90	92	89	85
μ_{app}		0.306	0.302	0.306		0.303		0.291	0.290	0.255	0.215
k_d		-0.005	0.000	-0.005		-0.002		0.011	0.012	0.047	0.087
k_{df}		0.000	0.001	0.000		-0.001		0.001	0.001	0.05	-0.010
DS, $\mu\text{g mL}^{-1}$											
		6.25	12.5	25		50		100	150	200	300
$C_f \times 10^{-5}$		6.3	6.4	6.3		6.5		6.6	6.7	7.0	6.5
V		89	88	84		85		86	84	83	82
μ_{app}		0.296	0.302	0.298		0.300		0.312	0.316	0.339	0.312
k_d		0.005	0.000	0.004		0.002		-0.011	-0.014	-0.037	-0.011
k_{df}		0.0011	0.000	0.001		0.000		0.001	-0.001	0.023	-0.0002

Effect on final viable cell concentration (C_f), viability (V), maximum apparent specific growth (μ_{app}) and death (k_d) rates and death rates at the final part of the experiment (k_{df}). C_f is expressed in cells mL⁻¹, V as percentage of total viable cells, μ_{app} and k in day⁻¹. The corresponding values for the control cultures were $6.4 \cdot 10^5 \pm 3.5 \cdot 10^3$ cells mL⁻¹, $90 \pm 1\%$ and 0.3 day^{-1} for C_f , V , and μ_0 respectively. The values in the table are calculated from the time-evolution of the mean values of cell concentration and cell viability for the two replicas. In none of the cases the standard deviations of the means were higher than a 6% of the value of the mean

the Trypan Blue exclusion method. Apparent growth rates (μ_{app} , day⁻¹) in a time interval ($t_0 - t_f$) were calculated from the slope of the best fitting line of the natural logarithm of the non-dimensional viable cell concentration (N_{vf}/N_{i0}) versus time t (Kunas and Papoutsakis 1990; Michaels et al. 1996). For maximum apparent specific growth rates only portions of the growth curve showing exponential increase were used for calculations.

As previously defined by Kunas and Papoutsakis (1990) an apparent growth rate at a certain culture condition is considered to be the sum of an unchanging true growth rate (the growth rate of the corresponding control culture, μ_0) and a death rate, k (day⁻¹), which depends on the culture conditions. Therefore, death rates are calculated as:

$$k = \mu_0 - \mu_{app}$$

If the values of the specific growth rates are the maximum k will be designated k_d and if the growth rates are calculated at the final part of the experiments k will be designated k_{df} . Positive values of k will be obtained under growth-detrimental conditions and negative values will mean that cultures are carried out under growth-promoting conditions.

Viscosity of the cell membrane

To determine whether or not the additives used in this study had any effect on the viscosity of the plasmatic membrane, the molecular rotor 9-(2,2-dicyanovinyl)julolidine (DCVJ, Sigma-Aldrich, Ref. 72335) was used. DCVJ is a fluorescent molecular rotor with a quantum yield that is proportional to the viscosity of its environment (Haidekker et al. 2001). The different additives assayed were added to cells from static cultures and kept at an orbital shaking table, with a 1.9 cm shaking diameter, at 90 rpm during 30 min before addition of DCVJ. Cultures with no additives were used as control, and kept static before DCVJ addition. In this work, the protocol described in Mallipattu et al. (2002) was followed and adapted for use in flow cytometry. Briefly, 10 μ L of a 2.5 g L⁻¹ solution in methanol of DCVJ were added to 1.0×10^6 cells in 400 μ L PBS, incubated at room temperature and in the dark during 1 h. Afterwards, cells were washed twice with PBS and analyzed by flow cytometry (Coulter Epics XL-MCL), exciting

DCVJ at 488 nm and measuring the emission between 505 and 540 nm.

Adaptation to freely-suspended culture

Se301 cells were cultured at 27 °C in 50 mL Erlenmeyer flasks, with a working volume of 6.5 mL, and at a relatively high initial density of approximately 0.5×10^6 cell mL⁻¹ in order to avoid limitations on the growth (Wu et al. 1990). The cultures were carried out in an orbital shaker with an orbital diameter of 1.9 cm at different shaking speeds (60, 70, 90 and 120 rpm). Previous direct cell adaptation without the addition of additives was attempted at 70, 90 and 120 rpm (Reynolds numbers of 2,555, 3,285 and 4,381, respectively). However, it was not successful due to severe clumping and a sharp decrease in the viable cell population during the first 72 h. Thereafter, a progressive adaptation process was adopted. It consisted in maintaining the cultures at a fixed shaking speed with the addition of additives until a constant growth rate during 4 generations was achieved. In this way, the shaking speed was increased step by step from 60 to 70, 90 and 120 rpm ($Re = 2,190, 2,555, 3,285$ and 4,381, respectively) as cells were adapted to the new shaking conditions until no cell damage was observed (Cruz et al. 1998). Before subculturing, broths were allowed to settle for 15–20 min in order to remove clumps, and to collect the supernatant with the single cells to be later subcultured, as described by Rhiel et al. (1997). As shaking speed was increased, cultures already growing as single cells tended to form clumps to a lesser degree, and these were easier to dissolve. The adaptation process was completed using combinations of the above-mentioned protective and disaggregant additives added at different, non-toxic concentrations (see Table 2). The cells were subcultured every 3 or 4 days when the cell density reached a value of around $1.5\text{--}2.0 \times 10^6$ cell mL⁻¹, to prevent the cultures from entering the stationary phase, where sensitivity to shear stress is higher (Goldblum et al. 1990). The adaptation process length ranged between 50 and 80 days.

Growth was measured by cell counts from samples collected before each passage from each culture. The total volume of culture was maintained constant by replenishing the volume removed for analyses with fresh medium.

Table 2 Apparent growth rates and death rates corresponding to suspended Se301 cell cultures carried out under several combinations of protective and disaggregant additives at different concentrations and turbulence levels

Block	Assay	<i>N</i> , rpm	<i>Re</i>	τ (N m ⁻²)	Protective additive, %w/v		Disaggregant, $\mu\text{g mL}^{-1}$		μ_{app} or μ_0 , day ⁻¹	k_d , day ⁻¹
I	Control	0	0	0	–	–	–	–	0,209 ± 0,094	
	C1	90	3285	5.79×10^{-4}	–	–	–	–		$> \mu_{app}$
	C2	120	4381	1.30×10^{-3}	–	–	–	–		$> \mu_{app}$
II	1	90	3584	6.21×10^{-4}	PF68	0.10	–	–	0.036 ± 0.110	0.173
	2	90	3584	6.21×10^{-4}	PF68	0.10	DS	25	0.135 ± 0.103	0.074
	3	90	3584	6.21×10^{-4}	PF68	0.10	DS	50	0.135 ± 0.111	0.074
	4	90	3584	6.21×10^{-4}	PF68	0.10	DS	100	0.168 ± 0.089	0.042
III	5	90	3285	5.79×10^{-4}	CMC	0.15	–	–	0.016 ± 0.100	0.193
	6	90	3285	5.79×10^{-4}	CMC	0.15	DS	25	0.069 ± 0.149	0.140
	7	90	3285	5.79×10^{-4}	CMC	0.15	–	–	0.029 ± 0.078	0.181
	8	120	4381	1.30×10^{-3}	CMC	0.15	–	–	0.039 ± 0.001	0.170
IV	9	90	3584	6.21×10^{-4}	PEG	0.10	DS	25	0.116 ± 0.121	0.093
	10	90	3584	6.21×10^{-4}	PEG	0.10	DS	50	0.176 ± 0.090	0.033
V	11	90	3584	6.21×10^{-4}	PVP	0.10	DS	25	0.165 ± 0.068	0.044
	12	90	3584	6.21×10^{-4}	PVP	0.10	DS	50	0.117 ± 0.049	0.092
	13	90	3584	6.21×10^{-4}	PVP	0.10	DS	100	0.214 ± 0.056	–0.005
	14	90	3584	6.21×10^{-4}	PVP	0.20	DS	25	0.125 ± 0.040	0.084
VI	15	90	3584	6.21×10^{-4}	PVP	0.20	DS	50	0.179 ± 0.068	0.030
	16	90	3285	5.79×10^{-4}	PVA	0.10	–	–	0.115 ± 0.051	0.094
	17	90	3285	5.79×10^{-4}	PVA	0.10	DS	25	0.166 ± 0.089	0.042
	18	90	3285	5.79×10^{-4}	PVA	0.10	DS	50	0.038 ± 0.101	0.172
	19	90	3285	5.79×10^{-4}	PVA	0.10	DS	100	0.226 ± 0.043	–0.017
	20	90	3285	5.79×10^{-4}	PVA	0.20	DS	25	0.200 ± 0.042	0.009
	21	120	4381	1.30×10^{-3}	PVA	0.10	DS	25	0.184 ± 0.067	0.025
	22	120	4381	1.30×10^{-3}	PVA	0.20	DS	25	0.213 ± 0.080	–0.004

The values of μ_{app} are the means of the growth rates of three successive passages once cultures have been adapted to suspension or at the end of the culture if adaptation was not possible. Error values correspond to the standard deviation of the means. Growth rate corresponding to the static control is defined as μ_0 . The values of k_d , as defined in [Materials and methods](#), are the difference between the average values of μ_0 and μ_{app} . Turbulence is characterized by the Reynolds number (*Re*), the shaking rate (*N*) and the shear stress (τ). Depending on the additive used, results have been divided into six different blocks (I–VI)

The values of the shear rate and Reynolds were calculated with the equations described in García Camacho et al. (2007) from the characteristics of the medium, the culture flasks and the agitation speed.

The viscosity of suspensions was measured using a viscosimeter (DV-II + Pro, Brookfield Engineering, Middleboro, MA). Data of shear stress (N m⁻²) versus shear rate (s⁻¹) exhibited a Newtonian behaviour in the range of Reynolds numbers. The apparent viscosity was 1.10×10^{-3} Pa s for the PF68, PEG and PVP supplemented media and 1.20×10^{-3} Pa s for the CMC and PVA supplemented ones. The bulk density

of the suspensions was measured using a pycnometer ($1,010.5 \text{ kg m}^{-3}$) (García Camacho et al. 2007). Changes in physical properties during culture were determined not to be significant.

Infection with the baculovirus SeMNPV

Both static and freely-suspended Se301 cell cultures were infected with the budded form of SeMNPV. The infection was carried out at different multiplicities of infection (MOI): 0.003, 0.007, 0.043, 0.5 and 1.0 for static cultures (*Re* = 0), and 0.003, 0.007, 0.012,

0.019, 0.207 and 1.0 for suspended ones ($Re = 3285$). Cells in the mid-exponential growth phase were used to prevent inhibition of viral production by excessive cell concentration (Agathos 1996), or possible nutrient limitations (Maranga et al. 2003). Viral titer (of budded virus) was determined with the end point dilution assay (EPDA) as described by King and Possee (1992). Eight days after infection cells were lysed to release the occluded viruses produced. To this end, a sample of 1 mL was taken from each culture and subsequently sonicated using a HP200S sonicator (Hielscher Ultrasonics GmbH, Teltow, Germany). The sonotrode was introduced into the culture, which was sonicated during 2 min. Because this process generates a huge amount of heat, samples were kept on ice. After cell lysis, the occluded virus produced were counted using an haemocytometer (Neubauer chamber).

Results and discussion

Influence of the protective and disaggregant additives on the growth of the Se301 cell line

When an additive is added to a cell culture it might inhibit, stimulate or be neutral to cellular growth. To determine this extreme with the Se301 cell line, assays were conducted with the additives listed in the **Materials and methods** section at the different concentrations listed in Table 1.

In Table 1 are summarized the results of final cell concentration (C_f), cell viabilities (V), maximum specific apparent growth rates (μ_{app}), specific death rates (k_d , calculated in the exponential growth phase), and death rates calculated in the final part of the experiments (k_{df}) (see Fig. 1 as an example). Considering the definition of k_d and k_{df} , when k_d is positive the additive shows fast-acting toxic effects and if k_{df} is positive the additive shows cumulative toxic effects. Comparing k_d and μ_{app} values, and taking into account cell viabilities, none of the additives except for PF68 were toxic at low concentrations. PF68 showed lethal effects ($k_d \geq \mu_{app}$) once the concentration of the additive was $>0.2\%$ w/v. PVA, PVP and CMC were slightly toxic at the two highest concentrations assayed (0.8 and 1.0%). PF68 and PVP showed only a fast-acting toxic process

Fig. 1 Time evolution of the average cell concentration of the control and the PVA-added, at 1%, culture as an example of the calculation of k_d and k_{df} . k_d is calculated in the exponential growth phase from the difference of the maximum specific growth rate of the control culture (μ_0) minus the maximum apparent growth rate of the additive-added culture. k_{df} is calculated in the same way but with the growth rates after the exponential growth phase

(very low k_{df} values), CMC showed only a cumulative toxic process (low k_d and high k_{df}) and PVA showed a concentration-dependent combination of both toxic processes (k_d and k_{df} increase from 0.6 to 1%). DS promoted growth ($k_d < 0$) at the highest concentrations tested ($>100 \text{ mg mL}^{-1}$). The repeated detachment process can cause some cell damage and, as will be discussed later, the anticlumping nature of DS might have made the cells interact weakly with the culture surface and detach easily. As mentioned in Materials and methods section, CMC and PVA affect the viscosity of the medium and viscosity affects mass transfer (Kilonzo and Margaritis 2004). A poor oxygen mass transfer capacity of the medium and not toxicity could have caused the cell concentration decrease observed at the final part of the experiments (high k_{df} values). Taking this into account CMC would not be toxic (low k_d values at all concentrations) and PVA would be toxic at the highest concentrations assayed (high k_d values).

Both CMC and DS are ionic additives, while PVA, PVP and PF68 are non ionic. It is known that the additives interact with the cellular membranes (Goldblum et al. 1990; Zhang et al. 1992); and it has been found that the more hydrophobic the compound the more toxic is its nature (Neimert-Andersson et al. 2006). This could explain the toxic nature of the non-

ionic additives used. PF68 is the most widely used and studied protective additive in shear sensitive cell cultures. It has been observed that, besides being incorporated to the plasmatic membrane decreasing its fluidity (Palomares et al. 2000), it can enter and accumulate within the cells (Gigout et al. 2008). The wide range of cellular processes that this molecule can trigger makes its influence on the cellular metabolism dependent on the cell line and on the concentration used (Elibol 1999; Lee and Kim 2002; Hellung-Larsen et al. 2000). It is possible that the rest of non ionic additives (PVA and PVP), not as widely studied as PF68, at high concentrations have an effect similar to that observed with PF68.

Adaptation of the Se301 cell line to suspension culture

Table 2 summarizes the combinations of agitation intensity, additives used and the results obtained for μ_{app} and k_d in the assays aimed at the adaptation to freely-suspended culture. The concentrations of the additives assayed were selected according to the previous toxicity results summarized in Table 1. In the adaptation process two disaggregant additives were evaluated: heparin and dextran sulphate (DS). Heparin showed a limited effectiveness in preventing cell clumping and the results have not been included. Therefore, in this part of the experimental plan, the disaggregant used was DS. Se301 cells tended to clump when growing in suspension. To eliminate the largest clumps, they were allowed to settle and discarded. This procedure allowed that after 20–30 passages most of the Se301 cells were growing as single cells. This protocol was also followed by Gioria et al. (2007), which permitted the adaptation of the UFL-AG-286 cell line from *Anticarsia gemmatilis* to suspension cultures, even though large clumps were formed when agitation was initiated.

Protective and anticoagulant effects

Depending on the additive used, results in Table 2 have been divided into six different blocks (I–VI). In block I (Assays C1 and C2) no protective or disaggregant additives were added. Se301 cells show a high anchorage dependency for growth and attach very intensely to the culture surface. As mentioned above, in these two assays it was not possible to obtain stable

cultures in suspension without additives directly from attached cultures. Cells aggregated severely and died in less than 4 days. The high concentration of big clumps made it impossible to determine cell concentrations precisely and, therefore, did not permit to calculate k_d accurately. But, since cells died in few days, death rate must have been higher than growth rate. These two assays demonstrate that the Se301 cell line is very sensitive to shear stress. This could be explained by its anchorage dependency and its spindle-shaped cells. Wu et al. (1990) justified the poor adaptation of Tn5 cells, an anchorage-dependent and spindle-shaped cell line, to growth in suspension based on their cell geometry, which is not very favourable to agitation. Therefore, this type of cells would need, for their successful cultivation in suspension, firstly a disaggregant, and secondly a shear protectant to prevent cell damage due to their not-rounded shape.

The additives in Table 2 with the most beneficial effect on the cell growth rate were PVP (Assays 11–15) and PVA (Assays 16–22), along with the addition of DS to reduce cell clumping. With some combinations of additives it was impossible to obtain stable cultures and standard deviations were, in some instances, higher than the value of the growth rate. Of all the combinations assayed in Table 2, only three of them (Assays 13, block V; 19 and 22, block VI) stimulated slightly the cell growth ($k_d < 0$) above the additive-free static control culture. Since the Reynolds number, the protective additive and its concentration were different, the improvement observed in cell growth could not be attributed to only one but to the interaction of these three variables. Only with PVA and PVP almost no micro-clumps were observed. With the rest of additives clumping was evident at various degrees, being CMC the one that most promoted clumping.

Fluid-dynamic conditions

Maximum values of τ assayed (see Table 2) were below $1.39 \cdot 10^{-3} \text{ N m}^{-2}$, much lower than those reported to be critical to other cell lines such as Sf21 ($1\text{--}4 \text{ N m}^{-2}$), Sf9 ($0.59\text{--}1.5 \text{ N m}^{-2}$), and *T. ni* TN-386 (0.1 N m^{-2}) (Tramper et al. 1986; Goldblum et al. 1990; Cruz et al. 1998). Assay 22 in Table 2 showed a growth rate similar to the static control at the maximum agitation speed tested. This would indicate that cells could be adapted to higher shear stress levels with no foreseeable cell damage.

Membrane fluidity

The staining experiments with DCVJ demonstrated that PVA, PVP, PEG and PF68, at all additive concentrations assayed, interact with the cellular membrane of Se301 cells because a shift in the fluorescence intensity of DCVJ was observed. This shift, indicates that the membrane fluidity of Se301 cells decreased by the action of the additive, i.e. making the membrane more rigid. It was further found that the additive-cell membrane interaction was strong: in some experiments cells were washed twice with PBS after the incubation with the protective additives and before the staining with DCVJ. In these assays the fluorescence intensity also increased with respect to the static additive-free cells, indicating that the additive was still present on the cell membrane. Having a more rigid membrane makes cells more resistant to shear stress (Zhang et al. 1995). As other authors suggest, the additive-membrane interaction is a fast process (less than 30 min) and, therefore, the protective effect observed in the results in Table 2 cannot be attributed to a physiological effect (Goldblum et al. 1990).

Why DS has shown to be successful with PVA, PVP and PEG and not with PF68 is not clear? It could be that the amphiphilic nature of PF68 (high hydrophilic-hydrophobic balance number) (Bassetti 1995) confers to PF68 the capacity to interact with cell membranes in such a way that inhibits or prevents the interaction with DS.

In this process, a possible adaptation expressed as selection of a conformational clone more resistant to shear stress has been rejected, since cell shape in suspended cultures was the same as in the static ones.

Infection with the baculovirus SeMNPV

Amongst all the additives used in this work, PVA confers the maximum protection against shear stress, at two different combinations (Assays 19 and 22). From the point of view of using the lesser amount of additives in a bioprocess, the combination of additives of Assay 19 would be preferable. But, since it has been documented that DS addition might inhibit the baculovirus infection (Dee et al. 1997b), the concentration corresponding to assay 22 would be preferable since it is the lowest DS concentration that

Fig. 2 In vitro OB production from Se301 cells infected with SeMNPV at 5 different MOIs in static and agitated ($N = 90$ rpm, $Re = 3285$) cultures. Agitated cultures were supplemented with the protective additive PVA at 0.2% w/v and the disaggregant DS at $25 \mu\text{g mL}^{-1}$. Horizontal and vertical bars represent the standard deviations of the means of MOI and OB concentrations for the 5 experiments, at each agitation condition, below and above MOI 0.1, respectively

allowed clump dissolution (Assay 22: [PVA] = 0.2%, [DS] = $25 \mu\text{g mL}^{-1}$).

Due to variability observed in the cell permissiveness to infection, the ten different experiments could be grouped in two groups, $\text{MOI} < 0.1$ and $\text{MOI} \geq 0.1$, in order to obtain a clear pattern in the effect of turbulence and MOI. The means of the values of OB concentrations below and above the MOI threshold value (0.1) were calculated. These means are shown in Fig. 2 with the corresponding standard deviation (bars). Final OB concentration increased with MOI (> 0.1), but no clear influence of agitation was observed.

The optimal MOI generally depends on each virus-insect cell line system (Micheloud et al. 2009). Our results diverge from those in which enhanced productivities of both heterologous proteins and viruses are frequently observed at relatively low MOIs (< 0.1) (Enden et al. 2005).

Conclusions

In this work, the adaptation to suspension, which is the first step of an insect-cell based bioprocess, of a highly attachment-dependent cell line has been carried out with success. A progressive adaptation of the Se301 insect cell line to grow as freely

suspended cultures in serum-containing medium has been achieved using both protective reagents against shear stress and disaggregant additives. The addition of these additives proved to be necessary in order to achieve freely suspended cultures of Se301 cells. Cell growth without additives was not possible, even though shear stress values to which they were subjected were within the range of non-lethal values to most insect cell lines published. PVA-DS or PVP-DS were the combinations of additives that promoted growth levels similar or higher to those in control static cultures and led to a virtually complete disaggregation of the cell clumps. It has been confirmed that the possible cause of cell adaptation was a more rigid cell membrane caused by the fast interaction additive-cell membrane.

The strategy proposed is recommended for other animal cell lines sensitive to shear stress. Although specific experimentation is the only way to collect truly handy quantitative data on the influence of shear stress on cell growth.

In this work, both static and freely-suspended Se301 cell cultures were infected with the autochthonous SeMNPV from Almería (Southeastern Spain). Final OB concentration increased with MOI (>0.1).

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