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RESEARCH ARTICLE

Evaluation of concentrated milk whey as a supplement for SF9 *Spodoptera frugiperda* cells in culture

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Keywords: baculovirus, concentrated milk whey, Grace's medium, insect cells, media formulation, *Spodoptera frugiperda* Sf9.

Abbreviations:
FBS: fetal bovine serum
FBS: fetal bovine serum
MOI: multiplicity of infection
MWR: milk whey ultrafiltration retentate
MWU: milk whey ultrafiltrate
PF68: Pluronic F68
YE: yeast extract

Insect cell culture has become increasingly useful for the production of heterologous proteins as well as of baculovirus polyhedra, and several different culture media formulations can be employed for this purpose. The goal of this work was to assess the potential of lyophylized milk whey ultra filtration retentate (MWR) when associated to yeast extract (YE), glucose and Pluronic F68 (PF68) to partially replace fetal calf serum

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(FBS) in *Spodoptera frugiperda* Sf9 cells culture in Grace's medium, aiming AgMNPV baculovirus production. Batch cultivation results showed that the yeast extract and the milk whey concentrate effectively increased cell concentration to about half the level commonly verified for the Sf900II serum-free medium. A 4-fold increase in viable cell concentration was achieved when employing 5% (w/v) MWR, 8 g/L YE, 1% (v/v) of FBS and 2.7 $g.l^{-1}$ of glucose, resulting in 1.6 x 10^7 $polyhedra.ml^{-1}$ after infection with baculovirus.

In the last decade the insect cell/baculovirus system has been widely used to produce wild-type baculoviruses as biopesticides and for the abundant expression of recombinant proteins. In this system, the protein post-translational modifications are similar to those found in mammalian cells (Verma et al. 1998). In addition, insect cells present several advantages when compared to mammalian cells, such as ease of culture, higher tolerance to osmolality (Olejnik et al. 2003) and much higher expression levels when infected with recombinant baculovirus (Ikonomou et al. 2003). The use of baculovirus as an alternative pesticide in agriculture has increased steadily in the last years. In 1992 it was estimated that 1.2 million litres of chemical insecticides were replaced worldwide by baculovirus preparations (Vardar-Sukan and Sukan, 1992). Recently, in several countries such as Brazil, baculovirus-based pesticides obtained through the infection of velvet bean caterpillar larvae were not used in larger quantities simply because of their limited supply. Since this tendency is increasing worldwide (Szewczyk et al. 2006), causing significant economical and environmental impact, strategies that allow the production of baculovirus at lower costs and higher amounts are particularly interesting. Approaches based on the infection of *in vitro* cultured insect cells with baculovirus may be economically attractive if culture media costs can be reduced, provided that virus infectivity is maintained after successive cultures.

Insect cell culture can be performed in basal media, such as Grace's medium supplemented with 5% or 10% (v/v) serum, most usually fetal bovine serum (FBS). However, problems such as lot-to-lot inconsistency, introduction of adventitious agents and presence of proteins may limit FBS widespread use in the insect cell/baculovirus system. Therefore, many studies have been performed during the last years aiming to reduce or eliminate serum requirements from insect cells, resulting in several effective serum-free formulations available in the market, as discussed by Ikonomou et al. (2003). On the other hand, in spite of serum-free media such as Sf900II being capable to support high cell densities, frequently their costs are too high when large scale baculovirus polyhedra production is desired. In addition, some of these media have still to be supplemented with lipids and yeast extract to result in adequate cell growth and virus production.

In Brazil, due to the extensive alcohol production by fermentation and cheese processing, residues such as yeast extract and milk whey are readily available, and could be used as inexpensive and highly nutritive insect cell culture media supplements.

Yeast extract contains amino acids, peptides, polysaccharides, vitamins, nucleic acids, lipids and traces of important metals that can promote Sf9 cell growth. Increases in its concentration in IPL-41 insect cell culture medium from 4 to 8 $g.l^{-1}$ can result in cell densities as high as 18 x 10^6 $cells.ml^{-1}$ in continuous culture (Drews et al. 1995).

Milk and milk whey, on the other hand, have been used as nutritional components in fermentation media not only for lactic acid bacteria (Bury et al. 1998), but also for normal or transformed fibroblasts (Steimer and Klagsbrun, 1981), for established cell lines (MDCK, Vero, CV-1, NRK, 3T3, F2408, and NIL8) and early passage cell strains (bovine articular chondrocytes, bovine smooth muscle cells, human foreskin fibroblasts, and rat embryo cells) (Steimer et al. 1981), for mouse hybridoma (Ramírez et al. 1990), and also for insect cells (Batista et al. 2005). Batch cultivation of *Spodoptera frugiperda* Sf9 cells in Grace's medium containing 2.7 $g.l^{-1}$ glucose, 8 $g.l^{-1}$ YE and 0.1% (w/v) PF68 supplemented with 1% (w/v) of milk whey ultra filtrate and 3% (v/v) FBS increased viable cell concentration to about 5-fold when compared to Grace's containing 10% (v/v) FBS, while AgMNPV polyhedra (PIBs) production after cell infection was around 3-fold higher in the supplemented medium than in Grace's with 10% FBS (Batista et al. 2005). Among other components, milk whey contains proteins, lactose and lipids, being much less complex than FBS and,

Table 1. Average composition of the supplements lyophilized yeast extract and milk whey retentate (adapted from Sgarbieri et al. 1999 and Borges et al. 2001).

Component	Percentage of dry weight	
	YE[a]	MWR[b]
Proteins	48.6	83.8
Lipids	0.4	4.5
Carbohydrates	25.6	ND
Ashes	11.7	2.8
Fibers	3.3	ND
Lactose	ND	8.9

[a]Yeast extract with 25% of maltodextrin.
[b]Milk whey retentate.
ND – not determined.

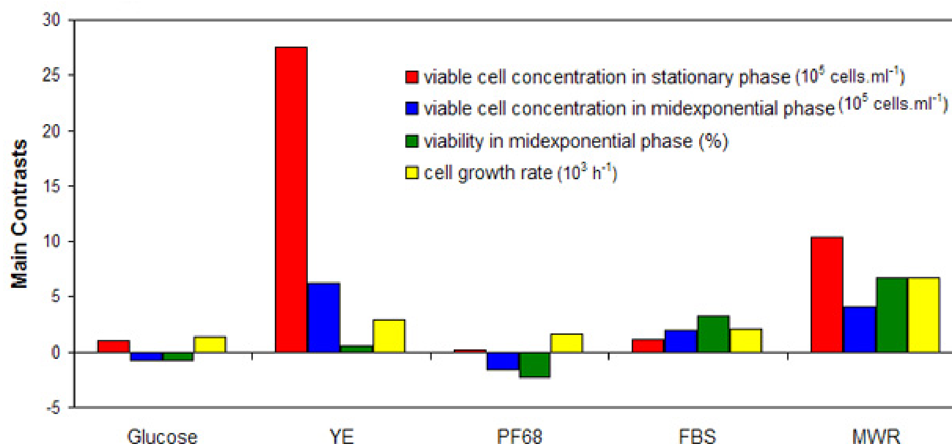

Figure 1. Main statistical contrasts on viable cell concentration in midexponential phase (X_{me}), viable cell concentration in stationary phase (X_s), viability in midexponential phase (V_{me}) and cell growth rate (μ) determined through the 2^{5-1} fractional factorial design for glucose, yeast extract (YE), Pluronic F68 (PF68), fetal bovine serum (FBS) and milk whey retentate (MWR). Cells were inoculated in different media at a final concentration of 2.5×10^5 cells.ml^{-1} and cultivated at 28ºC.

therefore, easier to be standardized when required, what motivated the present study.

The aim of this work was, therefore, the evaluation of supplements to Grace's medium, partially substituting FBS, intending to increase the productivity of *Spodoptera frugiperda* Sf9 cells and also of baculovirus, in continuation of other works previously published by the group (Batista et al. 2005; Rodas et al. 2005). The effects of glucose, Pluronic F68, yeast extract, concentrated milk whey and FBS on viable cell concentration in stationary and midexponential phase, on cell viability in the midexponential phase, and also on specific cell growth rate, were evaluated. The results herein discussed show that the addition of concentrated whey protein and yeast extract significantly increases cell concentration and also provides viral production comparable to that achieved in Sf900II medium at a much lower cost.

MATERIALS AND METHODS

Cell line, media and virus

Sf9 cells derived from *Spodoptera frugiperda* CRL 1711 (ATCC) were cultured in Sf900II medium (Gibco) or in Grace's medium (Gibco) at the Laboratory of Viral Immunology, Butantan Institute (São Paulo, SP, Brazil). The Grace's medium supplements tested were glucose (Gibco BRL), Pluronic F68 (Sigma Chemical Co.), FBS (Gibco BRL) and lyophilized yeast extract containing 25% maltodextrin, as well as milk whey ultra filtration retentate (both kindly donated by Dr. Valdemiro C. Sgarbieri, School of Food Engineering, State University of Campinas, Campinas, SP, Brazil). The milk whey derivative was obtained by ultra filtration employing a 10 kDa pore exclusion membrane (Borges et al. 2001; Pacheco et al. 2002) and its average composition, along with YE's, is given in Table 1. The *Anticarsia gemmatalis* nucleopolyhedrovirus AgMNPV employed was provided by Dr. Ronaldo Z. Mendonça.

Inoculum preparation

Stock cultures of Sf9 cells were stored in liquid nitrogen. After thawing, around 1.8 ml of cell suspension were transferred to 4 ml of Sf900II medium and cultivated in 40 mL Nunc flasks for 24 hrs at 28ºC. The spent medium was substituted with 4 mL of fresh Sf900II medium and cells were further cultured for 72 hrs. After cell release by vigorous manual agitation and transfer to Schott flasks, Grace's medium containing 10% (v/v) FBS was added. Cells were passed twice in this media for adaptation, and after centrifugation at 500 rpm for 30 sec, the pellet was resuspended in Grace's medium.

Cultivation procedures

All experiments were carried out in 100 mL shake flasks (with working volumes of 15 ml), inoculated with 2.5×10^5 viable cells.ml^{-1}, incubated in a rotary shaker at 100 rpm and 28ºC, similarly as performed by Batista et al. 2005. The analysis of the effects resulting from the addition of glucose, YE, MWR, PF68 and FBS to Grace's medium was performed employing a 2^{5-1} fractional factorial design, in a total of 16 experiments, as shown in Table 2. Grace's medium containing 10% (v/v) FBS was employed as a control. The performed fractional factorial design allowed the identification of the most important independent variables on cell proliferation, indicating the most appropriate variables to perform a further medium optimization study through an expanded full factorial design, aiming a quadratic model which could mathematically describe the system behaviour. This second study consisted d of a 2^3 trials experiment, with the central

Table 2. 2^{5-1} fractional factorial experiment for supplement screening. Cells were inoculated in different media at a final concentration of 2.5 x 10^5 cells.ml^{-1} and cultivated at 28ºC.

Assay	Independent Variables					Response Variables			
	GLU[a] (g.l^{-1})	YE[b] (g.l^{-1})	PF68[c] (%)	FBS[d] (%)	MWR[e] (%)	X_s[f] (x10^5 viable cells.ml^{-1})	X_{me}[g] (x10^5 viable cells.ml^{-1})	V_{me}[h] (%)	μ[i] (h^{-1})
1A	1.8	0.0	0.0	1.0	5.0	21.3	9.6	99	0.0230
2A	2.7	0.0	0.0	1.0	0.0	2.1	1.7	75	0.0064
3A	1.8	8.0	0.0	1.0	0.0	40.4	14.9	90	0.0141
4A	2.7	8.0	0.0	1.0	5.0	44.1	10.2	93	0.0223
5A	1.8	0.0	0.2	1.0	0.0	3.3	2.5	84	0.0069
6A	2.7	0.0	0.2	1.0	5.0	20.7	7.8	95	0.0223
7A	1.8	8.0	0.2	1.0	5.0	35.4	10.7	91	0.0247
8A	2.7	8.0	0.2	1.0	0.0	39.6	8.5	96	0.0223
9A	1.8	0.0	0.0	3.0	0.0	5.0	4.1	99	0.0223
10A	2.7	0.0	0.0	3.0	5.0	19.2	9.4	98	0.0231
11A	1.8	8.0	0.0	3.0	5.0	41.8	17.0	99	0.0165
12A	2.7	8.0	0.0	3.0	0.0	36.5	13.0	92	0.0161
13A	1.8	0.0	0.2	3.0	5.0	23.2	9.6	98	0.0187
14A	2.7	0.0	0.2	3.0	0.0	6.4	4.3	86	0.0167
15A	1.8	8.0	0.2	3.0	0.0	36.6	8.4	87	0.0187
16A	2.7	8.0	0.2	3.0	5.0	47.0	15.8	98	0.0239
Grace's+FBS	0.7	-	-	10.0	0.0	9.0	4.0	80	0.0266

Data presents the ranges of the independent variables [a]glucose,[b]yeast extract, [c]Pluronic F68, [d]fetal bovine serum, and [e]milk whey retentate on the dependent variables [f]viable cell concentration in stationary phase, [g]viable cell concentration in midexponential phase, [h]viability in midexponential phase and [i]specific cell growth rate.

point performed in triplicate, plus a set of tests in star expansion configuration, as shown in Table 3. Viable cell concentration at the stationary phase (X_s) was one of the response variables in both studies, as well as viable cell concentration (X_{me}) and viability in midexponential phase (V_{me}), the last two measured at half time between the beginning of cell growth after full cell adaptation and the end of cell logarithmic growth phase, since the midexponential growth phase seems to be the most adequate period for insect cell infection with baculovirus (Power et al. 1994).

The effects of the independent variables on the response variables were estimated through the software Statistica 5.0 (StatSoft Co.).

Viral production

When required, before viral infection, cells were adapted to the tested media formulations by a stepwise change of the medium in T-25 tissue culture flasks, typically taking 2 to 3 weeks for a complete medium change. The cells were sub cultured in the selected media at an inoculation density of 3

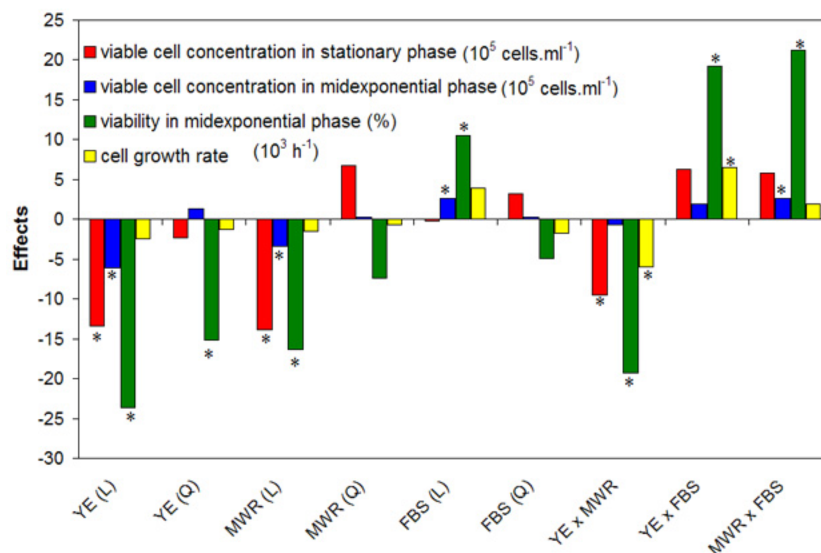

Figure 2. Statistical effects of yeast extract (YE), milk whey retentate (MWR) and fetal bovine serum (FBS) at a confidence level of 90% obtained for the 2^3 star expanded full factorial design on viable cell concentration in midexponential phase (X_{me}), viable cell concentration in stationary phase (X_s), viability in midexponential phase (V_{me}) and cell growth rate (μ). Cells were inoculated in different media at a final concentration of 2.5 x 10^5 cells.ml^{-1} and cultivated at 28ºC. *Significant effects at 90% confidence level. (*L*) refers to linear terms in the mathematical model. (*Q*) refers to quadratic terms.

x 10^5 cells.ml^{-1}, and were considered adapted when, after at least 6 sub cultivations, confluence was observed.

Twelve serial dilutions were performed for titrating the viral stock samples. Each diluted viral stock sample (100 µL) was seeded into 96-well plates containing 1 x 10^5 Sf9 cells.ml^{-1}per well. The plate was incubated at 28ºC for seven days, when the viral titer was calculated through the standard method (50% tissue-culture infectious dose, $TCID_{50}$). Cells grown in the 100 ml shake flasks (with a working volume of 15 ml) were infected at a concentration of 1 x 10^6 cells.ml^{-1}, at a multiplicity of infection (MOI) of one, which was determined by $TCID_{50}$, and incubated for six days, when baculovirus polyhedra production was analyzed.

Analytical methods

Cell concentration and viability were evaluated through optical microscopy (Olympus, model CK2) with Trypan Blue. Measurements of lactate, glucose and glutamine concentrations were carried out using a 2700 YSI biochemical analyzer (Yellow Spring Instruments), while ammonia content was determined through a 95-12 Orion probe analyzer coupled to a SA720 Procyon potenciometer. Media osmolalities were analyzed with an osmometer (Osmette A Precision System, Inc.) and viral polyhedra concentration was determined by counting in a hemocitometer by optical microscopy

Determination of specific substrate consumption and metabolite production rates

The specific rates of substrate consumption (glucose and glutamine) and metabolite formation (lactate and ammonia) q_i were determined during the exponential growth phase from equation 1:

$$q_i = \frac{dS_i/dt}{dX_v/dt} * \mu \qquad [1]$$

in which S_i is the concentration of substrate or metabolite *i*, μ is the apparent specific cell growth rate, X_v is the viable cell concentration, and *t* is time.

RESULTS AND DISCUSSION

Screening design for the evaluation of the effects of glucose, PF68, YE, MWR and FBS on X_s, X_{me}, V_{me} and μ

Previous performed work (Batista et al. 2005) has shown that, in Grace's media enriched with glucose, YE and the shear protective agent Pluronic F68, lyophilized milk whey ultra filtrate (MWU), a supplement rich in lactose) can be successfully employed to enhance Sf9 cells growth, partially substituting FBS. In the present work, the influence of the complementary fraction of the milk whey ultra filtration process (the retentate) on cell development was assessed, along with the effects of other important supplements.

To simultaneously identify the statistical effects of the tested supplements on cell growth and the possible interactions between the several different supplements, a

Table 3. 2^3 Complete factorial design experiment for YE, MWR and FBS supplements. Cells were inoculated in the different media at a final concentration of 2.5 x 10^5 cells.ml^{-1} and cultivated at 28º C.

Assay	Independent Variables			Response Variables			
	YE[a] (g.l^{-1})	MWR[b] (%w/v)	FBS[c] (%v/v)	X_s[d] (x10^5 viable cells.ml^{-1})	X_{me}[e] (x10^5 viable cells.ml^{-1})	V_{me}[f] (%)	μ[g] (h^{-1})
1B	4	3	1	35.2	12.9	91	0.0147
2B	8	3	1	34.2	10.1	88	0.0141
3B	4	5	1	37.5	10.1	91	0.0198
4B	8	5	1	0.0	0.0	0	0.0000
5B	4	3	3	31.1	13.4	92	0.0216
6B	8	3	3	25.0	8.5	78	0.0182
7B	4	5	3	27.5	9.8	85	0.0187
8B	8	5	3	20.1	9.7	82	0.0150
9B	2.6	4	2	37.5	19.5	92	0.0141
10B	9.4	4	2	14.0	5.4	62	0.0154
11B	6	2.3	2	54.6	13.2	94	0.0154
12B	6	5.7	2	22.4	8.7	82	0.0138
13B	6	4	0.3	33.1	7.9	90	0.0138
14B	6	4	3.4	34.2	13.8	93	0.0138
15B	6	4	2	29.2	9.8	86	0.0169
16B	6	4	2	20.1	8.0	84	0.0141
17B	6	4	2	25.0	9.5	88	0.0133
Grace's + FBS	-	-	10	14.7	8.1	96	0.0231
Sf900II	-	-	-	41.0	14.6	66	0.0198

Data presents the ranges of the independent variables [a]yeast extract; [b]milk whey retentate and [c]fetal bovine serum used to optimize the dependent variables [d]viable cell concentration in stationary phase, [e]viable cell concentration in midexponential phas, [f]viability in midexponential phase and [g]specific cell growth rate.

fractional design experiment was performed. Table 2 presents results for the 2^{5-1} fractional factorial design experiment in which the influence of glucose, PF68, YE, MWR and FBS on X_s, X_{me}, V_{me} and μ was evaluated and compared to Grace's control medium. X_{me} values varied from 1.7 x 10^5 to 17.0 x 10^5 viable cells.ml^{-1} and V_{me} reached high levels, varying from 75% up to 99%. X_s varied from 2.1 x 10^5 to 47.0 x 10^5 viable cells.ml^{-1} and μ varied from 0.0064 h^{-1} to 0.0266 h^{-1}, demonstrating clearly the importance of adequate nutrient supply. The calculated statistical contrasts of the independent variables on the response variables are shown in Figure 1. The variables that presented higher positive influence on viable cell concentration at the midexponential growth phase as well as on cell viability during this culture period and also on final cell concentration were YE concentration followed by MWR and FBS percentage. PF68 had a small effect on the evaluated response variables. Increasing glucose

concentration did not result in significant influence on the response variables in the tested range.

Assays 3A, 4A, 7A, 11A, 12A and 16A showed the best results concerning Sf9 cell concentration at the midexponential growth phase. With these formulations, cell concentration was from 2.6 to 4.3 times higher than the value observed for the control experiment with Grace's medium supplemented with 10% FBS.

Based on the fact that YE, milk whey retentate and fetal bovine serum presented the most important effects on cell growth, a supplementary study was proposed to determine the optimum supplement concentration range for cell concentration and viability as well as for specific cell growth rate, using a star expanded full factorial design. Since PF68 is a cell-protecting agent against shear damage normally employed at large scale cell culture, in this set of tests this compound was maintained, however at a concentration of 0.1% to reduce costs. Glucose concentration, on the other hand, was maintained at the highest level (2.7 $g.l^{-1}$) to prevent growth limitation due to its early consumption, since no catabolic repression was observed at this substrate concentration. The independent variables selected were, therefore, YE, MWR and FBS for the subsequent study.

Extended studies on the effects of YE, MWR and FBS on X_s, X_{me}, V_{me} and μ

Table 3 shows the results for the star expanded full factorial design experiment for YE, MWR and FBS. The estimated statistical effects of the independent variables on the response variables are shown in Figure 2. Decreasing YE and MWR concentrations resulted in increases in X_s, X_{me}, V_{me} and μ. Increasing FBS concentration, on the other hand, resulted in a significant increase in cell viability at a confidence level of 90%. Interestingly, in medium formulation 4B, cell growth was inhibited.

The interactions between YE and FBS and also between MWR and FBS were significant at a confidence level of 90%, revealing positive effects on X_s, X_{me} and V_{me}. However, the interaction term between YE and MWR at a confidence level of 90% showed significant negative effects on X_s and V_{me}. The *p*-value shows that most of the quadratic terms involving YE, MWR and also FBS were not statistically significant, therefore, a mathematical

Table 4. Kinetic and economic parameters for Sf9 cells in selected media after cell adaptation. Cells were inoculated in media 4A, 16A, 9B and 11B at a concentration of 3 x 10^5 $cells.ml^{-1}$ and cultivated at 28ºC and 100 rpm.

Parameter	Formulation 4A	Formulation 16A	Formulation 9B	Formulation 11B	Grace's + 10% FBS	Sf900II control
Viable cell concentration at midexponential phase (10^5 $cell.ml^{-1}$)	13.8	14.0	6.3	5.8	5.8	18.0
Viable cell concentration at stationary phase (10^5 $cell.ml^{-1}$)	37.5	34.2	23.0	27.5	9.5	80.0
Specific cell growth rate (h^{-1})	0.014	0.020	0.016	0.021	0.015	0.028
Population doubling time(h)	50	35	43	33	47	25
Glucose specific uptake rate (10^{-10}g/cell x h)	4.0	5.6	3.1	4.6	2.1	4.5
Glutamine specific uptake rate (10^{-11}g/cell x h)	2.0	2.9	0.9	0.0	5.0	4.5
Lactate specific consumption rate (10^{-11}g/cell x h)	3.4	4.9	12	44	98	-5.7[a]
Ammonia concentration on day 12 ($mg.l^{-1}$)	4.2	ND	4.5	6.1	5.1	66.2
Osmolality ($mOsm.kgH_2O^{-1}$)	408	427	379	400	326	350
Media cost (US$ per liter)	38.30	38.80	34.80	30.20	23.00	44.00

[a] Negative sign indicates production.
ND – not determined.

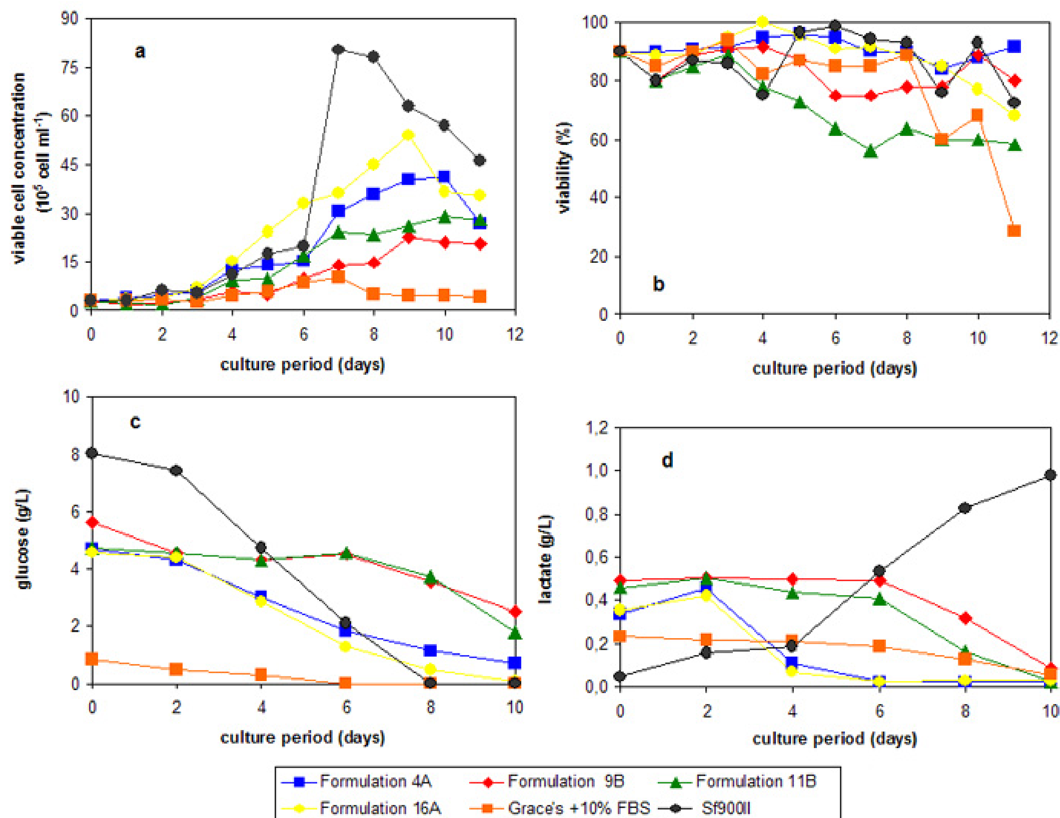

Figure 3. Kinetic behaviour of Sf9 cells in selected media after cell adaptation. Sf9 cells cultures were performed using the selected media 4A, 16A, 9B and 11B. Cells were inoculated in the different media at a final concentration of 3 x 10^5 cells.ml^{-1} and cultivated at 28ºC.
(a) Viable cell concentration.
(b) Cell viability.
(c) Glucose concentration.
(d) Lactate concentration.

square model could not be efficiently used to explain the variations of cell concentration with glucose, MWR and FBS concentrations. However, the data show clearly that Sf9 cells can be satisfactorily cultivated in media supplemented with MWR and also that milk whey concentrate effectively increased cell concentration when compared to Grace's control medium supplemented with FBS. Medium with formulation 11B, containing 6 g.l^{-1} YE, 2.3% (w/v) MWR, 2% (v/v) FBS, 0.1% (v/v) PF68 and 2.7 g.l^{-1} of glucose, resulted in the largest viable cell concentration at the stationary growth phase, with a value even higher than that achieved for the expensive serum free synthetic Sf900II medium.

Cell culture adaptation to selected media

Medium formulation 11B and also other media compositions which resulted in appropriate compromise concerning cell performance involving high X_S, X_{me}, V_{me} and μ namely media 4A, 16A and 9B, were selected for cell adaptation further studies. The results achieved after six consecutive passages are shown in Figure 3. Serum-free media such as Sf900II have been developed containing significantly high concentrations of key nutrients, such as glucose and glutamine, and therefore, it was expected that this cell line would grow to higher cell densities in Sf900II medium than in serum-supplemented cultures. Nevertheless, cell densities achieved, along with other important kinetic parameters determined for the different media formulations, were similar to or higher than those in serum-containing Grace's medium, as shown in Table 4, proving that Sf9 cells can successfully grow in Grace's medium supplemented with FBS percentages lower than 3% v/v.

After adaptation in the proposed media formulations, viable cell concentration at midexponential phase increased only in formulation 4A when compared to the performed first-passage studies. While in formulation 16A a mild decrease was observed, in formulations 9B and 11B, X_{me} was significantly reduced.

Concerning viable cell concentration at the stationary phase, in formulation 4A a 15% reduction was observed

when compared to the prior first passage studies, while in formulations 16A, 9B and 11B the reductions were even larger, equal to 27, 39 and 50%, respectively.

When compared to Grace's control supplemented with 10% FBS, after adaptation, cell concentration during the midexponential phase in the proposed formulations increased from 5.8 to 14 x 10^5 cells.ml^{-1} and therefore a raise up to 2.4 times was achieved. Viable cell concentration at the stationary phase was even further increased, reaching values up to 3.9-fold the value observed for Grace's control medium. However, the highest cell density was observed in Sf900II medium. Cell viability above 85%, on the other hand, was maintained only for formulation 4A during 11 days.

In Grace's control medium, cells grew exponentially until glucose depletion, and remained in the stationary growth phase for four days. Glucose concentration was continuously depleted in all media during cell culture. The main product from its metabolism, lactic acid, was more intensively consumed in formulations 4A and 16A during the first culture days, while in the remaining formulations, its consumption was verified after the sixth cultivation day, except for Sf900II, in which lactate accumulated during the whole culture period, as previously reported by other groups (Palomares and Ramírez, 1996; Mendonça et al. 1999).

Glutamine was less expressively consumed in formulations 9B and 11B. In all formulations except for Sf900II, ammonia concentration reached values around 4.2 to 6.1 mg.l^{-1}, indicating that inhibitory levels were not reached. This behaviour was also observed by Mendonça et al. (1999), who noticed that ammonia concentration increased continuously and reached 2.6 mM at the end of the culture for Sf9 cells in batch culture without significant inhibitory effects.

The results obtained for Sf9 cell concentrations with the selected media are, however, lower than the ones resulting from the use of TNM-FH medium (Mendonça et al. 1999; Yamaji et al. 1999). This medium consists basically of Grace's medium supplemented with 10% of SFB, 3.33 g.l^{-1} of YE and 3.33 g.l^{-1} lactoalbumin hydrolysate, a milk derivative. According to Yamaji et al. (1999), the nutritional capacity of this medium is high enough to support the growth of 1.1 x 10^7 uninfected Sf9 cells x d/cm^3 in shake-flasks. The supplementation of TNM-FH with 0.33% of lactoalbumin hydrolysate allows the growth of up to 1.5 x 10^6cells.ml^{-1} (Mendonça et al. 1999).

Oxygen limitation is not normally verified in the conditions employed in this set of experiments. However, in Sf900II medium a higher cell concentration was observed, 8 x 10^6 cell.ml^{-1}. Probably, in this assay, the increase in lactate concentration, while glucose was still present, was associated to oxygen limitation. Therefore, in future works, glucose concentration should be preferentially kept around 2.7 g.l^{-1} to avoid premature exhaustion of this nutrient, since catabolic repression was not detected at this glucose concentration value.

The osmolality values observed for the proposed formulations are above Grace's and Sf900II control media osmolalities, which can be attributed to the composition of the supplements employed, described in Table 1. Media formulation 16A, for instance, in spite of including 70% less FBS than Grace's medium supplemented with 10% FBS, presents also YE and MWR, which are rich in proteins and ashes (both supplements), as well as in lactose (only MWR). These components are clearly able to cause large increases in media osmolality.

When referring to economical analysis, formulation 11B presented the lower cost, followed by formulations 9B, 4A and 16A. Although all formulations were more expensive than Grace's supplemented with FBS, they were also up to 31% less costly than Sf900II. The cost of the milk whey supplement was estimated to be around US$ 0.3 per gram, while the bulky prices of the control culture media and of the remaining media supplements employed were estimated by the industrial sector of Instituto Butantan, a Brazilian governmental company that works on biopharmaceuticals production.

Table 5. Polyhedra production in formulations 4A, 11B, Grace's medium supplemented with 10% FBS and Sf900II medium. Cells were inoculated at a concentration of 3 x 10^5 cells.ml^{-1} and cultivated at 28ºC and 100 rpm. Sf9 cells at a final concentration of 1.0 x 10^6 cells.ml^{-1} were infected with baculovirus during the midexponential phase, at a MOI of 1, and further cultivated at 28ºC.

Parameter	Medium formulation			
	4A	11B	Grace's + 10% FBS	Sf900II
Baculovirus produced (10^6 polyhedra.ml^{-1})	16.0	6.6	6.1	18.0
Cost for 10^{10} polyhedra (US$)	23.94	45.76	37.70	24.44

Polyhedra production by Sf9 cells

Since it was observed that FBS can successfully be partially substituted for yeast extract and milk whey ultrafiltration retentate, Sf9 cells cultivated in the selected media (4A and 11B) were infected with baculovirus at a multiplicity of infection (MOI) equal to one. These media were selected aiming to evaluate polyhedra production in the formulation presenting the lower cost (11B) and in the one involving the lower amount of FBS (4A).

High polyhedra production was obtained for all selected media formulations, as shown in Table 5. Formulation 4A resulted in viral production directly comparable to that of the serum-free media Sf900II medium and the results show clearly that the use of medium 4A is not only interesting concerning virus production, but is also discretely more advantageous economically and besides, differently from Sf900II, it presents a known formulation. Provided that the costs of the supplements may be further reduced, so can be the price of the baculovirus polyhedra produced in formulation 4A.

In comparison to previously reported results (Batista et al. 2005) employing milk whey ultra filtrate (MWU) instead of milk whey retentate, in the first system, after adaptation in the evaluated medium formulation (Grace's medium supplemented with 2.7 $g.ml^{-1}$ glucose, 8 $g.ml^{-1}$ YE, 0.1% PF68, 3% FBS and 1% MWU), 4.7 x 10^6 $cells.ml^{-1}$ and 1.6 x 10^7 viral $polyhedra.ml^{-1}$ were obtained, whereas with the proposed 4A medium formulation the same viral production was achieved, with the advantage of using a lower amount of FBS than that previously employed.

CONCLUDING REMARKS

Glucose concentration and PF68 percentage did not significantly affect cell growth and viability in the evaluated range. Glucose was depleted, lactate was produced and after two days it was consumed simultaneously with glucose, and can be considered as an alternative carbon source. The independent variables that most influenced cell concentration were YE concentration and MWR percentage. After full adaptation of the cells to grow in shake flasks using the selected media, viable cell concentrations in stationary phase up to 37.5 x 10^6 $cells.ml^{-1}$ were attained when using Grace's medium containing 5% MWR, 8 $g.l^{-1}$ YE, 1% FBS and 2.7 $g.l^{-1}$ glucose. The infection of Sf9 cells grown on this medium formulation with baculovirus resulted in up to 1.6 x 10^7 $polyhedra.ml^{-1}$. This concentration is 2.6 higher than that obtained with Grace's medium supplemented with 10% of FBS and only 0.9 lower than that observed with the Sf900II serum-free medium. Therefore, the milk whey derivative evaluated is an effective supplement to Grace's medium, together with yeast extract and low amounts of FBS and glucose, to obtain viral products at lower costs. Qualitative constraints concerning viral infection have to be addressed, however, for effective biopesticide production. Provided that the costs of milk whey derivatives are reduced, so will be the final baculovirus cost.

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