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Enhancement of Sf9 cells and baculovirus production employing Grace's medium supplemented with milk whey ultrafiltrate

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Abstract

Animal cells can be cultured both in basal media supplemented with fetal bovine serum (FBS) and in serum-free media. In this work, the supplementation of Grace's medium with a set of nutrients to reduce FBS requirements in *Spodoptera frugiperda* (Sf9) cell culture was evaluated, aiming the production of *Anticarsia gemmatalis* nucleopolyhedrovirus (AgMNPV) at a cost lower than those for the production using Sf900 II medium. In Grace's medium supplemented with glucose, Pluronic F68 (PF68) and yeast extract (YE), the effects of FBS and milk whey ultrafiltrate (MWU) on cell concentration and viability during midexponential and stationary growth phase were evaluated. In spite of the fact that FBS presented higher statistical effects than MWU on all dependent variables in the first cell passage studies, after cell adaptation, AgMNPV polyhedra production was comparable to that in Sf900 II. Batch cultivation in Grace's medium with 2.7 g l^{-1} glucose, 8 g l^{-1} YE and 0.1% (w/v) PF68 supplemented with 1% (w/v) MWU and 3% (v/v) FBS increased viable cell concentration to about 5-fold (4.7×10^6 cells ml^{-1}) when compared to Grace's containing 10% (v/v) FBS (9.5×10^5 cells ml^{-1}). AgMNPV polyhedra (PIBs) production was around 3-fold higher in the MWU supplemented medium (1.6×10^7 PIBs ml^{-1}) than in Grace's medium with 10% FBS (0.6×10^7 PIBs ml^{-1}). This study therefore shows a promising achievement to significantly reduce FBS concentration in Sf9 insect cell media, keeping high productivity in terms of cell concentration and final virus production at a cost almost 50% lower than that observed for Sf900 II medium.

Introduction

Insect cells have been increasingly used for recombinant protein and biopesticide production. One of the most commonly employed cell line for the production of high-value heterologous proteins through the baculovirus expression system (BEVS) is the *Spodoptera frugiperda* Sf9 cell.

Animal cells normally require serum when cultivated *in vitro* using basal media, however this supplement consist of an undefined mixture of components that can vary from lot to lot, and

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potential adventitious contaminants can be introduced in the culture through its use. Also, protein purification is significantly more difficult in the presence of serum. Therefore, efforts aiming the reduction or even total elimination of serum requirements in insect cell culture are of interest.

Many studies have been performed with the purpose of improving insect cell culture media formulation (Wilkie et al. 1980; Mitsuhashi 1989; Wang et al. 1993; Drews et al. 1995; Schlaeger 1996; Vaughn and Fan 1997; Ikonomou et al. 2001; Maranga et al. 2003, Marteijn et al. 2003, among others). The efforts to eliminate bovine serum resulted in several commercially available serum-free media for insect cells, however, many of these media present very high costs and still require supplementation with other complex components such as protein hydrolysates. Hydrolysates or peptones are produced by enzymatic or chemical digestion of casein and albumin, yeast cells, plant and animal tissues, and basically consist of mixtures of peptides and aminoacids (Ikonomou et al. 2003). Some supplements, such as yeast extract, can also contain polysaccharides, vitamins, nucleotides, lipids and traces of important metals (Echalier 1997) and positively improve cell growth, as demonstrated by Lee and Park (1994) for Sf9 cells cultured in Grace's medium supplemented with yeastolate in the early stationary phase, achieving final cell densities up to 4-fold higher than when this compound was not added. In spite of the possible lot to lot variation of yeast extract, its quality consistency is much higher than that normally observed for FBS processing, and many standardized yeastolate formulations are commercially available, giving reproducible culture results.

Milk derivatives can also be successfully used as supplements for mammalian cells in culture (Ramírez et al. 1990), being non expensive, readily available, and much less complex than FBS. Hydrolyzed lactoalbumin, for instance, is an example of a milk derivative employed as a component of the TNM-FH insect cell culture medium. In spite of being an animal derived supplement and therefore possibly not suitable for the production of biopharmaceuticals using mammalian cells, milk derivatives might be an interesting alternative for the production of biopesticides. Also, being less complex than FBS, downstream processing of the target protein should be simpler when milk derivatives are used to substitute FBS.

The purpose of this work was therefore to evaluate, in *Spodoptera frugiperda* (Sf9) cell culture in Grace's media enriched with glucose, YE and the shear protective agent Pluronic F68, the potential of lyophilized milk whey ultrafiltrate (MWU, a supplement rich in lactose) as a cell growth enhancer, partially substituting FBS. The study aimed to increase Sf9 cells and AgMNPV polyhedra production when compared to the use of Grace's medium supplemented with 10% FBS culture media at a cost lower than that observed when Sf900 II medium is employed. The achieved results show that not only the addition of MWU significantly increased cell concentration (5-fold) after cell adaptation, but also that the viral product, polyhedra (PIBs), production augmented to about 3-fold the commonly observed value in Grace's medium containing 10% FBS at a cost round 50% inferior than that for Sf900 II.

Materials and methods

Virus, cell line, media and supplements

The *Anticarsia gemmatalis* nucleopolyhedrovirus AgMNPV and the Sf9 cells derived from *Spodoptera frugiperda* CRL 1711 (ATCC) used were maintained at the Laboratório de Imunologia Viral, Instituto Butantan (São Paulo, Brazil). Cells were cultured in Sf900 II medium (Gibco) or in Grace's medium (Gibco). The Grace's medium supplements were glucose (Gibco), PF68 (Sigma Chemical Co.), FBS (Gibco), lyophilized yeast extract containing 25% maltodextrin and milk whey ultrafiltrate (MWU) (both kindly donated by Dr. Valdemiro C. Sgarbieri, Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas, Campinas, SP, Brazil). MWU was obtained by ultrafiltration employing a 10 kDa pore exclusion membrane as detailed by Borges et al. (2001). The average compositions of the supplements MWU and YE are shown in Table 1.

Preparation of inocula

Frozen Sf 9 cells were thawed and propagated in Sf900 II medium in T-flasks (25 cm^2) at 28 °C.

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

Component	Percentage of dry weight	
	YE[a]	MWU[b]
Proteins	48.6	–
Lipids	0.4	–
Carbohydrates	25.6	–
Ashes	11.7	9.6
Fibers	3.3	–
Lactose	–	85.0

[a]Yeast extract with 25% of maltodextrin; [b]Milk whey ultrafiltrate.

After around 3×10^6 cells ml^{-1} were obtained, cells were passed twice in Grace's medium containing 10% FBS (v/v) for adaptation. The cells were centrifuged at 500 rpm for 30 s and the pellet was resuspended in Grace's medium in 100 ml shake flasks.

Experimental design for culture media formulation

The basal medium selected was Grace's medium due to its low cost, simple formulation and easy preparation at large quantities. A 2^2 factorial design scheme plus three replicates of the center point at the intermediate concentrations (shown in Table 2) was employed to study the effects of MWU and FBS on viable cell concentration in stationary (X_s) and mid exponential phase (X_{me}) or cell viability in the mid exponential phase (V_{me}), as well as on specific cell growth rate (μ). In all experiments Grace's medium was supplemented with glucose, yeast extract and Pluronic F68 to achieve final concentrations of 2.7 g l^{-1}, 8 g l^{-1}, and 0.1% (w/v), respectively. Both MWU and yeast extract were dissolved in Grace's medium and sterilized using a Millipore filtration unit. The range of concentrations selected for the supplements is in agreement with regular insect cell culture practice.

All experiments were carried out in 100 ml shake flasks inoculated with 2.5×10^5 viable cells ml^{-1}, with working volumes of 15 ml, incubated in an orbital shaker at 100 rpm and 28 °C. Grace's medium plus 10% (v/v) FBS and Sf900 II medium were used as controls for minimal and maximum culture media composition, respectively. The software Statistica version 5.0 (StatSoft Co.) was

Table 2. 2^2 complete factorial design experiment for MWU and FBS supplements.

Formulation	Independent variables		Response variables			
	MWU[a] (% m/v)	FBS[b] (% v/v)	X_s[c] ($\times 10^5$ viable cells ml^{-1})	X_{me}[d] ($\times 10^5$ viable cells ml^{-1})	V_{me}[e] (%)	μ[f] (h^{-1})
1	1.00	1.0	23.7	8.7	95	0.0130
2	2.50	1.0	16.8	6.4	94	0.0111
3	1.00	3.0	29.9	9.5	97	0.0135
4	2.50	3.0	23.3	9.3	98	0.0126
5	1.75	2.0	17.3	8.6	91	0.0091
6	1.75	2.0	16.9	8.6	93	0.0088
7	1.75	2.0	16.8	8.0	94	0.0088
Grace's + FBS	–	10.0	8.0	4.8	93	0.0239
Sf900 II	–	–	77.2	16.6	91	0.0301

Cells were inoculated in the different media at a concentration of 2.5×10^5 cells ml^{-1} and cultivated at 28 °C and 100 rpm.
Data present the ranges of the independent variables [a]milk whey ultrafiltrate; [b]fetal bovine serum used to optimize the dependent variables; [c]viable cell concentration in stationary phase; [d]viable cell concentration in midexponential phase; [e]viability in midexponential phase; [f]specific cell growth rate.

used to estimate the effects of the independent variables on the response variables.

Virus infection

Prior to viral infection, Sf9 cells were adapted to the selected medium by a stepwise change of the medium in T-flasks (25 cm^2), typically taking 2 to 3 weeks for a complete medium change. Cells were considered adapted when, after at least 6 subcultivations, confluence was achieved. For titration of viral stock samples, 12 serial dilutions were made. Aliquots of 100 μl of each diluted viral stock sample were seeded into 96-well plates containing 10^5 cells ml^{-1}. The plate was then incubated at 28 °C for 7 days and the viral titer calculated using the standard method (50% tissue-culture infectious dose, $TCID_{50}$). In the infection assays, cells which were grown in 100 ml shake flasks (with a working volume of 15 ml) were infected at a concentration of 10^6 cells ml^{-1}, at a multiplicity of infection (MOI) of 1. Cell samples were collected 6 days after infection to evaluate PIBs production.

Analytical methods

Cell concentration and viability were determined by optical microscopy (Olympus, model CK2), counting in Neubauer chambers using trypan blue (0.04%) exclusion and/or by fluorescence microscopy (Olympus, model BX 51) with acridine orange and ethidium bromide. Glucose, glutamine and lactate concentrations were measured using the 2700 YSI Biochemical Analyser (Yellow Spring Instruments). Ammonia was determined through a 95–12 Orion Probe Analyzer (Orion), and a SA720 Procyon potentiometer. Media osmolalities were determined using an osmometer (Osmette A Precision System, Inc).

Results and discussion

Effect of MWU and FBS on X_s, X_{me}, V_s and μ of Sf9 cells

The study consisted basically of two steps, one of them being the analysis of first passage cell behavior in several different medium formulations, and the second being the evaluation of cell performance after adaptation to the medium selected from the results achieved in the first step. The influence of MWU and FBS on cell growth and viability in the first passage of the cells in the different medium formulations tested was evaluated using a 2^2 experimental design with three replicates at the center point. The concentration range of MWU was established based on preliminary results that indicated cell growth inhibition at MWU concentrations above 2.5%, possibly due to the increase in the medium osmolality to deleterious levels. Since one of the purposes of this work was to evaluate MWU as a cell growth enhancer, partially substituting FBS in Sf9 cell cultures, the concentrations of FBS used were low and ranged from 1 to 3% (v/v). Table 2 summarizes the medium formulations evaluated and the results obtained.

Viable cell concentration for the proposed formulations in midexponential phase varied from 6.4×10^5 to 9.5×10^5 cells ml^{-1} and cell viability at this phase was between 91% and 98%. At the stationary phase, viable cell concentration in the different medium formulations varied from 16.8×10^5 to 29.9×10^5 cells ml^{-1}. Medium formulation 3 showed the second best results for cell growth and viability, following Sf900 II formulation.

The low specific cell growth rates observed in Table 2 are possibly related to the slow adaptation of the cells to the proposed formulations. However, the data also demonstrate that the new formulations based on Grace's medium could support the growth of Sf9 cells in reduced percentages of FBS.

Concerning the selected initial glucose concentration, previous results obtained at our laboratories (not shown) indicate that increases in glucose concentration in Grace's medium result only in a limited contribution to cell growth in the concentration range tested. However, it was also observed that the low glucose availability in Grace's medium causes a mild reduction in the length of the exponential cell growth phase, contributing to a reduction of the cell concentration in the stationary phase. This probably explains why in Grace's medium supplemented with FBS the final cell concentration was low. Therefore, the initial glucose concentration was maintained at 2.7 g l^{-1} in the proposed formulations to prevent

growth limitation due to the early consumption of this carbohydrate, since no catabolic repression was observed at this substrate concentration. This concentration value is similar to that observed in IPL-41 medium (2.5 g l^{-1}).

Pluronic F-68 was added to the tested medium formulations aiming to protect the cells from hydrodynamic stress when cultured in suspension and considering that it could increase recombinant protein production, even though maximum viral titers could be reduced as discussed in detail by Palomares et al. (2000).

The statistical effects of the independent variables on the dependent variables are shown in Figure 1. At a confidence level of 90%, the decrease in MWU concentration led to an increase in X_s, X_{me} and μ while the effect of MWU in association with FBS resulted in increases in all response variables analyzed. According to the statistical analysis, increasing FBS concentration resulted in positive effects on all dependent variables. This behavior was expected, since FBS is an agent stimulating cell growth. However, statistically significant effects were observed only for viable cell concentration during the midexponential phase (Table 3). According to non linear multiple regression and analysis of variance, the calculated F value was 24.4, being, therefore, more than 4 times higher than the listed value, equal to 5.93. The mathematical model generated given by Equation 1 (where C_{MWU} and C_{FBS} refer to the concentrations of MWU and FBS, respectively) was used to plot the response surface and contour diagram shown in Figure 2.

$$X_{me} = 8.44 - 0.63 * C_{MWU} + 0.93 * C_{FBS} + 0.53 * C_{MWU} * C_{FBS} \quad (1)$$

Therefore, independently of the tested MWU concentration, media containing high FBS percentages resulted in larger X_{me} values, and this dependent variable achieved its maximum for medium formulation 3. In addition, this medium resulted in an appropriated compromise concerning cell performance related to high values of X_S, V_{me} and μ.

Sf9 culture adaptation to selected medium and viral production

Assuming that if cells experienced too much difficulty to adapt in some media during the first passage, it is probable that those media would not

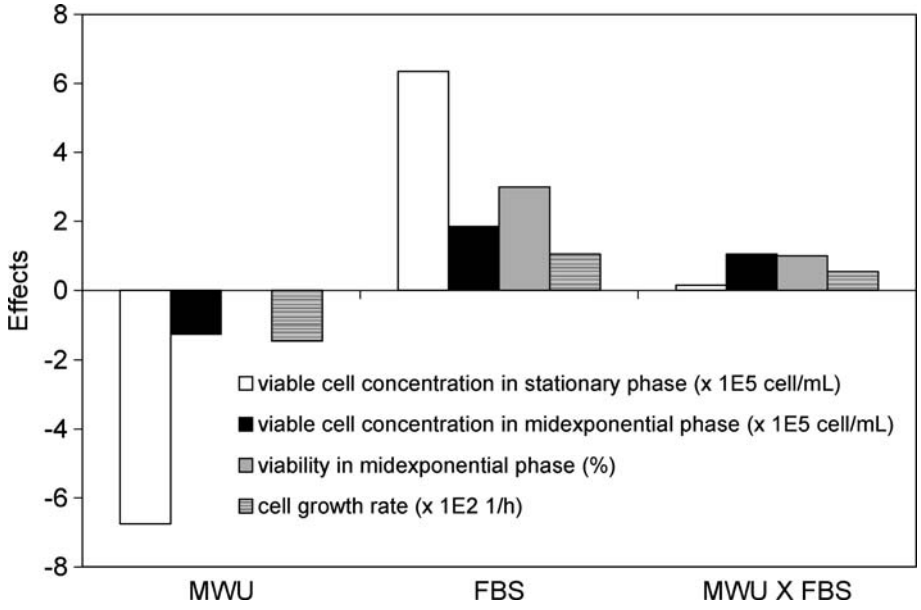

Figure 1. Statistical effects of milk whey ultrafiltrate (MWU) and fetal bovine serum (FBS) at 90% of confidence obtained for the 2^2 factorial design on viable cell concentration in midexponential phase (X_{me}), viable cell concentration in stationary phase (X_s), viability in midexponential phase (V_{me}) and cell growth rate (μ). Cells were inoculated in the different media at a concentration of 2.5×10^5 cells ml^{-1} and cultivated at 28 °C and 100 rpm.

Table 3. Statistical effects estimates on X_{me} from results of the 2^2 factorial design.

Factor	Effect	S.E.	$t(2)$	p
Mean	8.44	0.13	64.5	0.0002
MWU	− 1.25	0.35	− 3.61	0.0689
FBS	1.85	0.35	0.33	0.0333
MWU × FBS	1.05	0.35	0.09	0.0937

Cells were inoculated in different media at a concentration of 2.5×10^5 cells ml^{-1} and cultivated at 28 °C and 100 rpm.
All parameters obtained were statistically significant at 90% confidence level.

result in high cell or AgMNPV yield after cell adaptation. Thus, formulation 3 was selected for the remaining part of the study. Also, this formulation was further evaluated because, among the tested media, it resulted in the largest X_{me}, and since viral production was aimed and viral infection at moderate MOI values is preferentially performed at midexponential cell growth phase (Power et al. 1994), high cell concentration at this stage is desirable.

After adaptation, cell growth behavior in formulation 3 practically did not differ from the first passage results presented in Table 2. A 4.9 fold increase was attained in Sf9 cell concentration in the stationary phase with medium formulation 3 when compared to Grace' s medium supplemented with 10% FBS (Figure 3 and Table 4). Nevertheless, cell growth was still more elevated in Sf900 II medium, with cell concentration in the stationary

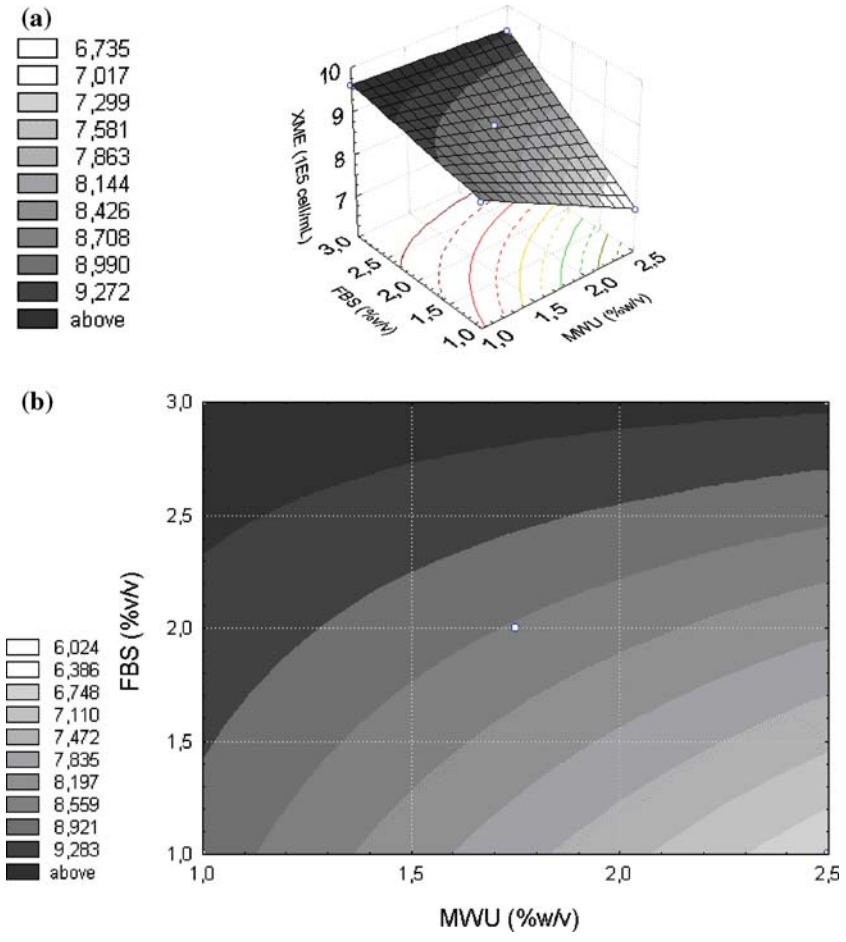

Figure 2. (a) Response surface and (b) contour diagram of viable cell concentration in midexponential phase (X_{me}) as a function of MWU and FBS concentrations. Cells were inoculated in different media at a concentration of 2.5×10^5 cells ml^{-1} and cultivated at 28 °C and 100 rpm.

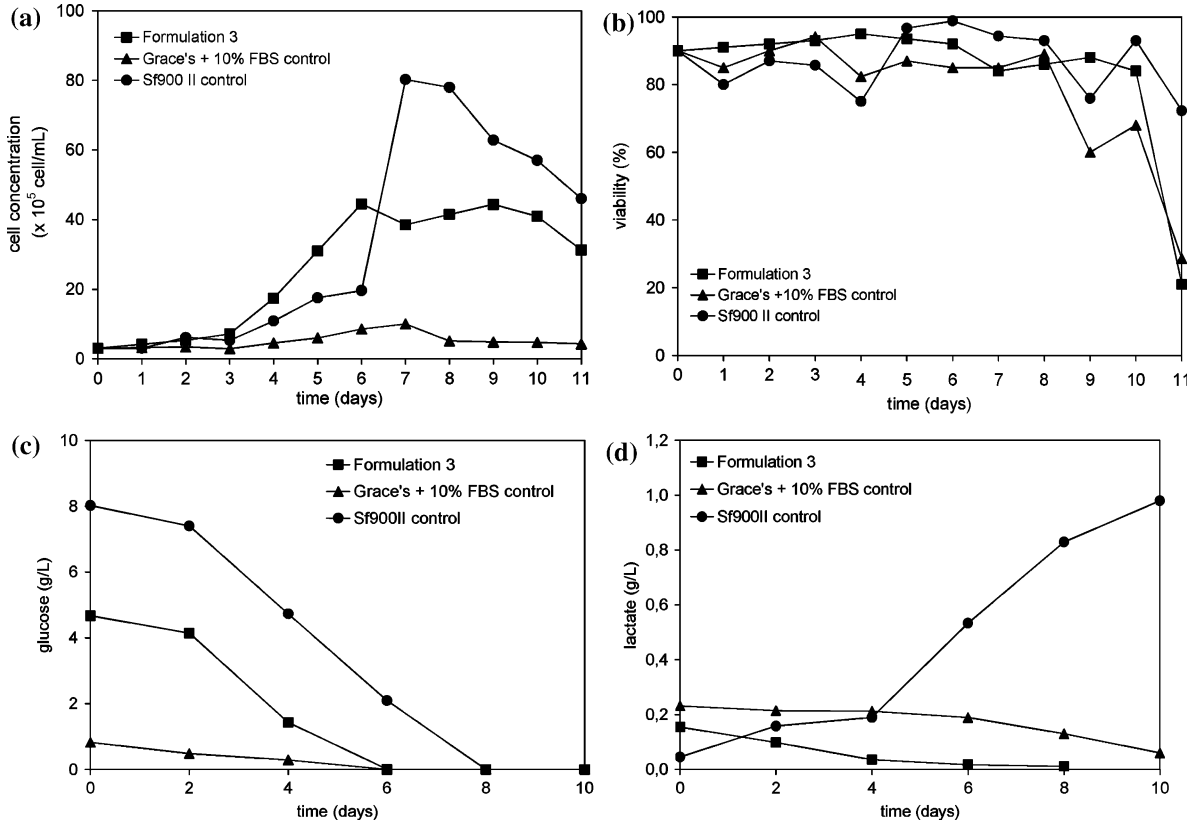

Figure 3. Kinetic behavior of Sf9 cells in formulation 3, Grace's supplemented with 10% FBS and Sf900 II media: (a) viable cell concentration; (b) cell viability; (c) glucose consumption; (d) lactate production. Cells were inoculated at a concentration of 3×10^5 cells ml^{-1} and cultivated at 28 °C and 100 rpm. Results were obtained after cell adaptation to the media tested during 6 successive passages.

phase reaching a value around 1.7 times higher than that observed for formulation 3.

During the first 10 days of culture, cell viability above 80% was maintained only in medium formulation 3. Viable cell concentration increased up to 46.7×10^5 cells ml^{-1} during the first 6 days of culture in this medium, showing that MWU stimulates cell growth intensively in the presence of low percentages of FBS when compared to the traditional Grace's medium containing 10% FBS. In this latter medium, cell viability decreased after 7 days of culture, when the stationary phase was established.

Since for insect cells culture, glucose is considered the most important carbohydrate (Ikonomou et al. 2003), its concentration was

Table 4. Kinetic parameters for Sf9 cells in selected media after cell adaptation.

Parameter	Media		
	Formulation 3	Grace's + 10% FBS	Sf900 II
Cell concentration at stationary phase ($\times10^5$ cells ml^{-1})	46.7	9.5	80.0
Cell growth rate (h^{-1})	0.021	0.015	0.028
Cell population doubling time (h)	34	47	25
Baculovirus produced ($\times10^6$ polyhedra ml^{-1})	16	6.1	18.0
Final ammonium concentration (mg ml^{-1})	5.5	5.1	66.2
Osmolality (mOsm kg^{-1} H_2O)	438	326	350

Cells were inoculated at a concentration of 3×10^5 cells ml^{-1} and were cultivated at 28 °C and 100 rpm.

monitored during cultivation. Glucose was continuously depleted in all tested media during cell culture, while lactic acid, the main product from its metabolism, strongly accumulated only in the Sf900 II control medium. Both in Grace's control and in formulation 3 media lactate accumulated to concentrations below deleterious levels, equal to 0.3 g l^{-1} according to Mendonça et al. (1999). Since glucose was still available in Sf900 II medium when lactate concentration increased, oxygen limitation probably occurred in this particular situation, as previously discussed by Mendonça et al. (1999). Upon glucose limitation, the lactate accumulated in Grace's medium (control) and in formulation 3 medium was consumed simultaneously to this carbon source, as previously reported by other groups (Bedard et al. 1993; Palomares and Ramírez 1996; Mendonça et al. 1999). Interestingly, total glutamine consumption was lower than 0.31 g l^{-1} in both formulation 3 and in Grace's control medium, however, it reached around 1 g l^{-1} in Sf900 II medium (data not shown).

Final ammonia concentration reached values up to 5.5 mg l^{-1} for formulation 3 medium and for Grace's control, as shown in Table 4, indicating that in these media inhibitory levels were not reached. Similar results were reported by Mendonça et al. (1999) for Sf9 cells in batch culture in TNM-FH containing 10% FBS (a culture medium that can be directly compared to both formulation 3 and Grace's control media), where ammonium concentration increased continuously, reaching 44 mg l^{-1} without significantly inhibitory effects. In Sf900 II medium, however, as shown in Table 4, higher levels of ammonium (above 60 mg l^{-1}) were detected at the end of the culture, but even at this higher value ammonium is probably not a cell growth inhibitor, since Sf9 cells can support concentrations of up to 180 mg ml^{-1} of NH_4^+ (Bedard et al. 1993).

The osmolality of medium formulation 3 was of 438 mOsm kg^{-1} H_2O, higher than that observed in Grace's and Sf900 II control media (326 and 350 mOsm kg^{-1} H_2O, respectively). In spite of including 70% less FBS than for Grace's medium which is supplemented with 10% FBS, formulation 3 medium presents also YE and MWU, which are rich in ashes (salts and trace elements in both supplements) and lactose (MWU), as presented in Table 1.

Considerable polyhedra production was obtained with formulation 3 medium (Table 4), which is comparable to that observed for the serum-free medium Sf900 II and which is 2.6 times higher than the viral production in Grace's control medium. Estimating MWU's cost to be around US$ 0.3 per gram, the calculated costs per liter of formulation 3 medium, Grace's medium supplemented with 10% FBS, and Sf900 II medium would be US$ 18.49, 23.00, and 44.22, respectively (the bulky prices of the control culture medium and of the remaining medium supplements employed were estimated by the industrial sector of Instituto Butantan, a Brazilian governmental company that works on biopharmaceuticals production). Hence, to achieve 16×19^9 polyhedra, one would spend US$ 18.49 if formulation 3 medium was employed, US$ 60.33 for Grace's medium supplemented with 10% FBS, and US$ 39.28 for Sf900 II medium, a result clearly favorable for the developed formulation 3 medium.

Conclusion

The achieved results show that MWU supplementation of Grace's medium combined with yeast extract, glucose and Pluronic F68 allowed not only a significant reduction in the percentage of FBS necessary to grow and maintain Sf9 cells, but also provided high viral productivity at competitive costs. After adaptation, up to 4.7×10^6 cells ml^{-1} and 1.6×10^7 viral polyhedra ml^{-1} were obtained with Grace's medium supplemented with 2.7 g ml^{-1} glucose, 8 g ml^{-1} YE, 0.1% PF68, 3% FBS and 1% MWR. The safety aspect of the animal source of MWU should not be an issue when baculoviruses to be employed as biopesticide are desired. In spite of limited safety, for recombinant protein production for instance, the use of this supplement instead of FBS should facilitate downstream processing. Further efforts have to be carried out, however, in view of total FBS elimination from the cell culture medium.

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