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DESIGN OF AN EFFICIENT MEDIUM FOR INSECT CELL GROWTH AND RECOMBINANT PROTEIN PRODUCTION

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SUMMARY

We report the development of a new serum-free medium based on the use of factorial experiments. At first, a variety of hydrolysates were screened using a fractional factorial approach with High-Five cells. From this experiment yeastolate ultrafiltrate was found to have, by far, the most important effect on cell growth. Furthermore, Primatone RL® was found to remarkably prolong the stationary phase of Sf-9 and High-Five cell cultures. The optimal concentrations for yeastolate and Primatone were determined to be 0.6 and 0.5%, respectively, on the basis of a complete factorial experiment. This new medium, called YPR, supported good growth of both Sf-9 and High-Five cells in batch cultures, with maximal densities of 5.4 and 6.1 × 10^6 cells/ml, respectively. In addition, both cell lines achieved good growth in bioreactor batch culture and had a prolonged stationary phase of 3–4 d in YPR medium compared to Insect-XPRESS medium. The ability of the new medium to support recombinant protein expression was also tested by infecting Sf-9 or High-Five cells at high density (2 × 10^6 cells/ml) with a baculovirus expressing secreted placental alkaline phosphatase (SEAP). The maximum total SEAP concentration after 7 d was about 43 IU/ml (58 mg/L) and 28 IU/ml (39 mg/L) for High-Five and Sf-9 cells, respectively.

Key words: hydrolysates; High-Five cells; Sf-9 cells; SEAP production; factorial experiment.

INTRODUCTION

Insect cell culture, particularly the culture of lepidopteran cell lines, has rapidly evolved into a large research effort since Grace established the first cell lines in vitro (Grace, 1962). The introduction of the baculovirus expression vector system (BEVS) in the early 1980s (Smith et al., 1983), for the production of heterologous proteins, has given a significant impetus to insect cell culture. Establishment of new cell lines (Vaughn et al., 1977; Hink et al., 1991; McKenna et al., 1998), advances in vector construction (Luckow, 1991; Possee, 1997), and metabolic engineering (Jarvis et al., 1998; Ailor and Betenbaugh, 1999), along with the development of serum-free culture for insect cells (Maiorella et al., 1988; Schlaeger, 1996a, 1996b) and large-scale production (Agathos, 1994, 1996), have transformed insect cell culture into a mature technology. Today, the insect cell/baculovirus system is widely used for the production of wild-type baculoviruses as biopesticides (Murhammer, 1996) and for the abundant expression of recombinant proteins.

The scale-up of this system requires large quantities of media. In particular, serum-free media are inexpensive, easy to prepare from individual components, suitable for both growth and infection phases, and capable of supporting high cell densities in different modes of operation (fed-batch, batch, or perfusion). Recently, the idea of using protein hydrolysates for the large-scale culture of animal cells has been gaining ground, especially in the context of total serum replacement (Jan et al., 1994; Schlaeger, 1996b; Heidemann et al., 2000). A serum-free medium for insect cell culture based on hydrolysates and referred to as IP301 medium (Schlaeger et al., 1993) comprised a defined formulation supplemented with 0.4% yeastolate, 5.0 g/L glucose, and a lipid–Pluronic F-68 emulsion. One-tenth of the powder needed for the IP301 would give the complete SF-1 medium when supplemented with 0.5% lactalbumin, 0.5% Primatone RL®, 0.4% yeastolate, and the above-mentioned emulsion. This low-cost medium could support growth of Sf-9 cells to 10^7 cells/ml in spinner flasks and in airlift bioreactor (23 L). Another serum-free medium, destined for the replication of *Autographa californica* nuclear polyhedrosis virus (AcMNPV), was reported (Vaughn and Fan, 1997). Amino acids were provided by a combination of 1% yeastolate, 0.5% tryptose, and 0.5% Primatone, with the exception of cystine and methionine, which had to be further supplemented. A *Spodoptera frugiperda* (Cl-15) and a *Lymantria dispar* (IPLB-Ld-FB) cell line were able to grow to high saturation densities in this medium. Moreover, the replication of AcMNPV was satisfactory in this medium (4.4 × 10^8 polyhedra/ml). Finally, medium development for the promising BTI-Tn5B1-4 (Tn-5) cell line (Wickham et al., 1992; Davis et al., 1993) resulted in the ISYL medium (Donaldson and Shuler, 1998). The latter was based on the IPL-41 basic medium, with 4 g/L Hy-Soy hydrolysate, 6 g/L yeastolate, 7.5 g/L glucose, and 2.5% lipid–Pluronic emulsion. Tn-5 cells reached a density of 6.1 × 10^6 cells/ml in batch culture in ISYL medium. This medium compared well with Ex-Cell 405 in terms of volumetric production of secreted human alkaline phosphatase (SEAP) (Donaldson and Shuler, 1998).

Thus, use of hydrolysates for the development of serum-free, low-protein or protein-free media is an attractive and promising alter-

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native to serum supplementation for insect cell culture. Yet, a thorough and rational screening of hydrolysates in this context has not been reported. Here we report the use of factorial experiments for initial screening and subsequent optimization of hydrolysate concentration in a serum-free medium capable of supporting the growth of the Sf-9 and High-Five (a clone of the BTI-Tn5B1-4 line) cells. The potential of our formulation to support protein production and large-scale culture is further demonstrated by bioreactor and shake-flask growth data and by the production of SEAP at high cell densities.

Materials and Methods

Cell lines and media. The Sf-9 and High-Five cells were a gift from Els Roode, Laboratory of Virology, Wageningen University, The Netherlands. The Sf-9 cells were cultured initially in Grace's medium supplemented with 10% fetal bovine serum (FBS). They were sequentially adapted to Sf-900 II serum-free medium in T-flasks and, afterwards, to suspension by gradual increase of the agitation rate. Subsequent adaptation to other serum-free media was quite easy with cells being completely adapted after two to three passages in the new medium. The High-Five cell line initially grew in aggregate form in serum-free culture. The cells were adapted to single-cell growth in suspension as follows: in the first phase of adaptation, which lasted 10 d, cells were passaged daily in Insect-XPRESS™ serum-free medium containing 10 U/ml heparin in 125-ml Erlenmeyer flasks with 25-ml liquid volume. In this phase cell concentration was kept between 10^6 and 2×10^6 cells/ml and agitation speed was increased from 120 to 140 rpm. In the second phase, heparin addition was stopped and cells were subcultured every 3 d. Inoculation was performed with cells coming mostly from the upper part of the culture. The whole procedure lasted 1 mo and resulted in full adaptation to single cell growth with occasional appearance of small (5–10 cells) aggregates. Similar adaptation procedures have already been reported (Rhiel et al., 1997) and they do not seem to negatively affect cell growth and recombinant protein production (Saarinen et al., 1999). Sf-9 cells for growth studies with Insect-XPRESS medium in shake flasks and bioreactor, as well as with Sf-900 II medium in shake flasks, were supplied by SmithKline Beecham Biologicals, Rixensart, Belgium, and had already been preadapted to serum-free, suspension culture. Both cell lines were maintained on an orbital shaker (model C24, New Brunswick Scientific, Edison, NJ) in 125-ml polycarbonate Erlenmeyer flasks (Corning Incorporated, Acton, MA) with 25-ml working volume at 27° C. The cells were subcultured every 3–4 d when viable cell density was between 2 and 3×10^6 cells/ml. Cells were counted with a Neubauer hemocytometer and cell viability was determined by trypan blue exclusion (0.2% final concentration). The media employed for routine maintenance and growth studies were: Insect-XPRESS (BioWhittaker Europe, Verviers, Belgium), Sf-900 II (Life Technologies SA, Merelbeke, Belgium), EX-Cell™ 400, EX-Cell™ 405, EX-Cell™ 420 (JRH Europe Limited, Buckinghamshire, UK), and HyQ®-SFX-Insect™ (Perbio Sciences NV, Erembodegem-Aalst, Belgium). Growth studies were attempted after a minimum of 10 passages in the respective serum-free medium was attained. Analyses of glucose, glutamine, ammonia, and lactic acid were done using a BioProfile 100 analyzer (Nova Biomedical, Waltham, MA). Amino acid analysis was performed by high-performance liquid chromatography (HPLC).

Design and preparation of YPR medium. For medium design and amelioration, IPL-41 and TC100 media, lactalbumin hydrolysate solution (50×), and tryptose phosphate broth (TPB) (50×) were from Life Technologies. Yeastolate ultrafiltrate (50×), glucose solution (45%), Pluronic F-68 solution (10%), and lipid mixture (1000×) for insect cell culture were purchased from Sigma Chemical Co. (St. Louis, MO). Primatone RL, HyPep 4601 and HyPep 4602 (wheat hydrolysates), HyPep 1510 (soy hydrolysate) and HyPep 5603 (rice hydrolysate) were a kind gift from Quest International (Naarden, The Netherlands). The Live/Dead Cytotoxicity Kit used to evaluate the toxicity of the plant protein hydrolysates was purchased from Molecular Probes (Leiden, The Netherlands). All experiments were performed in 250-ml shake flasks with 50-ml liquid volume, agitated at 150 rpm. Cells grown in Insect-XPRESS were transferred in flasks containing hydrolysate combinations and supplemented with glucose. Samples were taken daily for cell count and metabolite analysis.

The reagents used in YPR medium preparation for growth studies and maintenance of cell cultures were all purchased from Sigma, except Primatone RL (ICN Biomedicals, Asse-Relegem, Belgium) and IPL-41 medium in powder form (Perbio Science NV, Erembodegem-Aalst, Belgium). Large quantities of the medium were prepared by dissolving IPL-41 powder in MilliQ water and adding the hydrolysates, an emulsion of lipids in Pluronic F-68 solution and glucose and glutamine in powder form. $NaHCO_3$ (0.35 g/L) was subsequently added and the pH was adjusted to 6.2 by addition of 1 *N* NaOH. The medium was immediately sterilized using a Millipore filtration unit and kept at 4° C until use.

Bioreactor studies. A CelliGen Plus™ bioreactor (New Brunswick Scientific) of 1-L liquid volume was used for batch cultures of Sf-9 and High-Five cells in Insect-XPRESS and YPR media. The bioreactor was equipped with a pitched blade impeller, a pH probe (Broadley James, FermProbe model F-635), and an O_2 sensor (Mettler Toledo, 12/220 T-type). Cells for bioreactor inoculation grew in 500-ml shake flasks until a viable cell density of 2×10^6 cells/ml was reached or exceeded. Then, cells were gently centrifuged ($40 \times g$, 10 min), resuspended in 1 L of fresh medium, and transferred to the bioreactor. A shake flask serving as control was also inoculated. For all of the cultures, temperature was controlled at 27° C and dissolved oxygen (DO) level at 50% of air saturation by means of headspace aeration. Agitation rate was initially set to 80 rpm and progressively increased up to 110–120 rpm. Samples were taken every 24 h for cell count and metabolite analysis.

Infection studies. A recombinant *Autographa californica* baculovirus coding for the secreted human placental alkaline phosphatase (SEAP) was kindly provided by Dr. H. A. Wood, Boyce Thompson Institute for Plant Research, Ithaca, NY. For infection studies with the SEAP baculovirus, duplicate flasks of High-Five and Sf-9 cells in YPR medium were infected in mid-exponential phase at a density of 2×10^6 cells/ml with a multiplicity of infection (MOI) of 1. Complete change of medium was performed just before virus addition. Sampling was done at intervals of 24 h. Briefly, from 1200 μl of each culture, 200 μl were used for cell count. The other 1000 μl were centrifuged at 6000 rpm for 10 min and the supernatant was separated from the pellet. Both were kept at −20° C until the SEAP assay. SEAP concentrations (extracellular and intracellular) were determined by modifying the protocols of Cullen and Malim (1992) and Dee et al. (1997). After three cycles of freezing/thawing, cell pellets were resuspended in 1 ml of assay buffer (1 m*M* diethanolamine pH 9.8, 0.5 m*M* $MgCl_2$, 10 m*M* L-homoarginine) and heated to 65° C for 5 min to inactivate endogenous phosphatases. Cell debris were pelleted by centrifuging at 6000 rpm for 10 min and supernatants were appropriately diluted in assay buffer to be used for intracellular SEAP determination. Similarly, spent medium samples were thawed, heated to 65° C for 10 min, and diluted for extracellular SEAP analysis. From each sample, 10 μl were mixed with 1 ml of assay buffer and heated to 37° C for 10 min. Subsequently, 100 μl of *p*-nitrophenyl phosphate (120 m*M*) were added and the absorbance at 405 nm was measured at 1-min intervals for 10 min. For each sample, duplicate measurements were carried out. The slope (absorbance/min) was used to calculate SEAP activity in international units per milliliter as follows (Dee et al., 1997):

$$\frac{\text{IU}}{\text{ml}} = \frac{(\text{dilution factor}) \cdot (\text{slope}) \cdot 1110\ \mu\text{l}}{10\ \mu\text{l} \cdot (18.8\ \text{ml}/\mu\text{mol})}$$

Results

Experimental design. The first step in the development of our serum-free medium was the selection of the basic medium. For ease of preparation in large quantities, the basic medium should be available commercially in powder form. This was not the case for Grace's medium, which, in addition, contains organic acids and sucrose at high concentrations. The TC-100 medium exists in powder form, but it contains 0.26 g/L tryptose broth, which was one of the hydrolysates to be tested and it was quite difficult to filter. On the other hand, IPL-41 medium has a relatively simple formulation, and it is easy to filter, making it our choice of basal medium. We chose glucose as the major carbon source, as it is the most important carbohydrate for insect cell culture (Reuveny et al., 1992; Bédard

TABLE 1

2^{7-4} FRACTIONAL FACTORIAL EXPERIMENT FOR HYDROLYSATE SCREENING[a]

A	B	C	D	E	F	G	Treatment combination
−	−	−	+	+	+	−	def
+	−	−	−	−	+	+	afg
−	+	−	−	+	−	+	beg
+	+	−	+	−	−	−	abd
−	−	+	+	−	−	+	cdg
+	−	+	−	+	−	−	ace
−	+	+	−	−	+	−	bcf
+	+	+	+	+	+	+	abcdefg

[a] The designation for each hydrolysate along with the concentration corresponding to the "high" level are as follows: A = yeastolate ultrafiltrate (0.60%), B = lactalbumin hydrolysate (0.33%), C = tryptose phosphate broth (0.26%), D = Primatone RL (0.50%), E = HyPep 1510 (soy peptone) (0.40%), F = HyPep 5603 (rice peptone) (0.40%), G = HyPep 4602 (wheat peptone) (0.40%).

et al., 1993; Drews et al., 1995). Thus, IPL-41 medium was supplemented with glucose to a final concentration of 10 g/L (55.5 m*M*). As the medium is destined for both Sf-9 and High-Five cells, this high glucose level is justified by the significantly higher specific consumption rate of the latter during growth and infection (Rhiel et al., 1997). The seven hydrolysates that were initially screened are shown in Table 1. The concentrations for yeastolate ultrafiltrate, lactalbumin, TPB, and Primatone RL were those commonly reported. For the remaining three, a concentration of 0.4% was found to be nontoxic (Live/Dead Cytotoxicity Kit), and capable of supporting monolayer growth (data not shown). A lipid mixture containing cholesterol, cod liver oil, Tween 80, and D-α-tocopherol acetate (Maiorella et al., 1988) served as lipid source. We added 2.5× the recommended amount, i.e., a 400× instead of 1000× dilution of the lipid mixture, because this improves cell growth and final cell densities and may have a positive effect on secreted protein production as well (M. Donaldson, pers. comm.). Pluronic F-68 was employed as a shear protectant at a concentration of 0.1% (Murhammer and Goochee, 1988) and was emulsified with the lipid mixture before addition to the medium. Although the final medium should be suitable for the culture of both Sf-9 and High-Five cell lines, the latter cell line was employed for the factorial experiments. The reason for this was the rather high sensitivity of High-Five cells to hydrolysate quality (Danner et al., 1995), which could help to identify any adverse effects from the very beginning and to avoid the selection of hydrolysates with potentially toxic properties.

Hydrolysate screening and medium optimization. The best way to identify the effect of a certain hydrolysate on cell growth and any possible interactions between hydrolysates would be to perform a complete 2^k factorial experiment. In the case of seven hydrolysates, a total of 2^7 hydrolysate combinations would be necessary. As that was practically impossible, we rather chose to perform a 2^{7-4} fractional experiment, which would serve to estimate the main effects for all of them (Montgomery and Runger, 1999). Table 1 shows the design of the experiment where the absence or the presence (the "low" or the "high" level) of a hydrolysate in a certain combination is indicated by a "−" or "+" symbol. The final cell concentration was chosen as the response variable. The growth curves for the eight combinations along with the control (which is not taken into account in the effect calculation) are shown in Fig. 1A. It is worthwhile to note that all the flasks containing yeastolate ultrafiltrate had the highest final densities, two of them reaching the same densities as the control flask. Furthermore, glucose was consumed to a great extent or even completely in the flasks containing yeastolate (Table 2). There was a significant lactate accumulation for some hydrolysate combinations (Table 2). The main effects can be easily estimated by the following equation (Montgomery and Runger, 1999) (given for hydrolysate A as an example, similar calculation for the others):

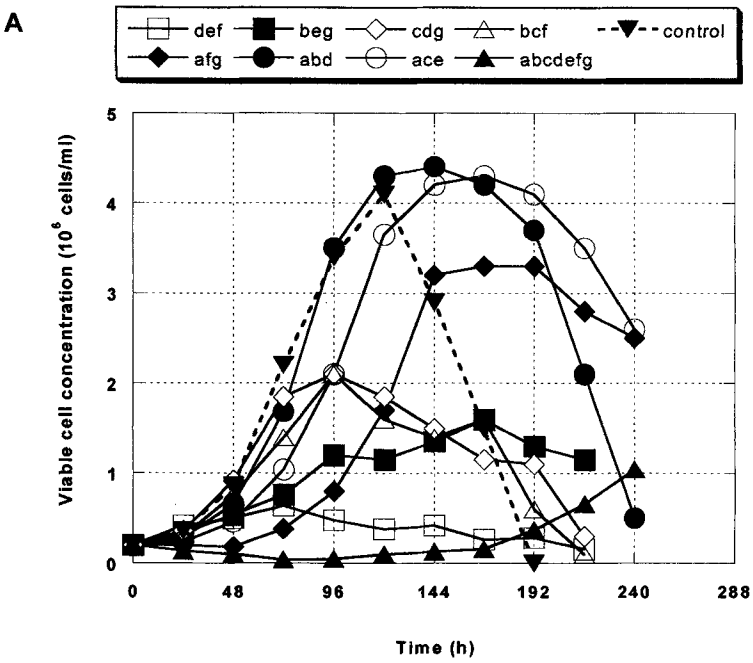
$$A = \overline{y_{A+}} - \overline{y_{A-}} = \frac{abd + afg + ace + abcdefg}{4} - \frac{def + beg + cdg + bcf}{4} \quad (1)$$

It can be seen in Table 3 that yeastolate ultrafiltrate had, by far, the most significant effect on final cell concentration. This effect cannot be attributed to its higher concentration compared to the other six hydrolysates, because a similar experiment with Sf-9 cells and yeastolate concentration of 0.4% gave similar results (data not shown). Therefore, yeastolate ultrafiltrate was the first hydrolysate to be chosen. The choice of a second peptone was based on the comparison of growth profiles (Fig. 1*A*) and metabolite data (Table 2). High-Five cells in the medium containing yeastolate, lactalbumin hydrolysate, and Primatone RL had almost identical growth profiles with the same cells growing in Insect-XPRESS medium. In addition, the culture in the "abd" combination was characterized by a prolonged stationary phase of 3 d, complete glucose consumption, and no lactate accumulation. Ammonia was produced at quantities normally found in Tn5 cell culture (Yang et al., 1996; Rhiel et al., 1997). We chose Primatone RL as the second hydrolysate, because apart from being a rich source of di- and tri-peptides it has been shown to exhibit antiapoptotic properties in mammalian cell culture (Schlaeger, 1996b).

The concentrations of the two hydrolysates, yeastolate ultrafiltrate and Primatone RL, were further optimized by a 2^2 complete factorial experiment. Two levels for yeastolate (0.4 and 0.6%) and Primatone (0.3 and 0.5%) concentration were tested. The presence or absence of the respective letter in Fig. 1*B* denotes the presence of a hydrolysate at its low or high level. The main effects of the two hydrolysates and the effect of their interaction on maximal cell concentration were calculated in a similar way as the effects in the fractional experiment (Table 3).

Once again, we see that yeastolate has the most important effect on cell growth. Furthermore, the presence of both hydrolysates at their "high" level (0.6 and 0.5%, respectively) results in the highest growth rate and final concentration. The medium was further supplemented with glutamine to reach a concentration of 10 m*M*, without taking into account the glutamine, free or in peptide form, coming from the two hydrolysates.

Addition of a further 10 m*M* asparagine, an amino acid which is required at high levels by High-Five cells (Danner et al., 1995; Rhiel et al., 1997), did not improve cell growth (data not shown). On the contrary, significant ammonia and lactate accumulation accompanied by growth inhibition occurred after 150 h (37 m*M* ammonia and 48 m*M* lactate versus 22 and 12 m*M* for the unsupplemented culture, respectively) (data not shown). Increase of Pluronic



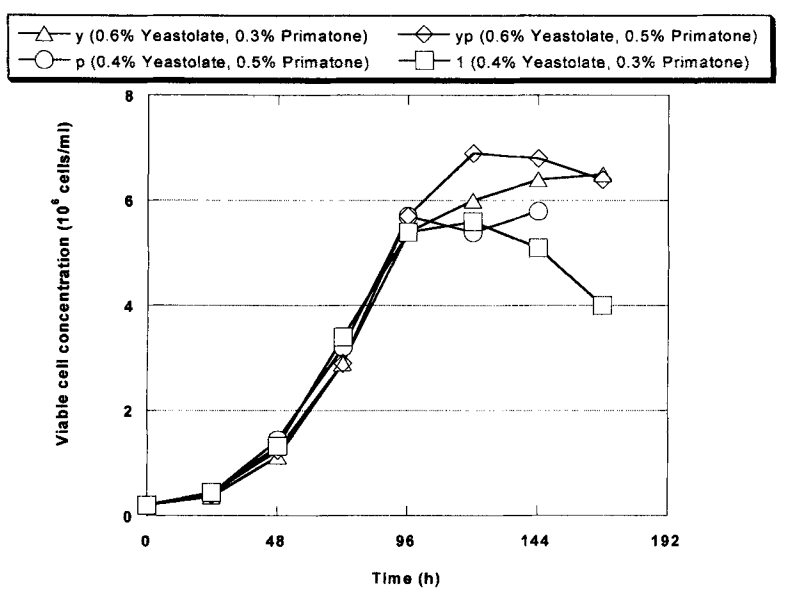


FIG. 1. (*A*) Fractional factorial experiment for hydrolysate screening. Hydrolysate combinations were added to IPL-41 medium containing 10 g/L glucose and a lipid–Pluronic emulsion. High-Five cells growing in Insect-XPRESS medium were transferred directly in each formulation. The control was Insect-XPRESS medium. (*B*) Complete factorial experiment for optimization of hydrolysate concentration. Experimental conditions same as in *A*.

F-68 concentration up to 0.4% neither inhibited nor promoted cell growth (data not shown). The final formulation of our medium, which is called YPR (Yeastolate–PRimatone), was the following:

- IPL-41 basic medium
- 10 g/L (55.5 m*M*) glucose
- 15–17 m*M* free glutamine
- 0.6% yeastolate ultrafiltrate
- 0.5% Primatone RL
- 0.1% Pluronic F 68–lipid mixture for insect cell culture (400× dilution)

The YPR medium was found to be quite rich in free amino acids as shown in Fig. 2. Alanine, which is not an essential amino acid for either Sf-9 or High-Five cells, comes mainly from yeastolate.

Cell growth in suspension cultures. The growth of Sf-9 and High-Five cells in YPR medium was compared to other serum-free media, as shown in Fig. 3. High-Five cells, after a lag phase of 1 d, grew to the highest final density (6.1×10^6 cells/ml) in YPR medium compared to 4.2×10^6 cells/ml in HyQ SFX-Insect and 4.5×10^6 cells/ml in EX-Cell 405 (Fig. 3*A*). The specific growth rate (and population doubling time [PDT]) for the exponential phase in the three media were $\mu = 0.032\ h^{-1}$ (PDT = 21.7 h), $\mu = 0.0318\ h^{-1}$ (PDT = 21.8 h), and $\mu = 0.0262\ h^{-1}$ (PDT = 26.4 h) for the YPR medium, HyQ SFX-Insect, and EX-

TABLE 2

METABOLITE CONCENTRATION IN HYDROLYSATE-CONTAINING MEDIA

Media formulation[a]	Glucose concentration (m*M*)		Ammonia concentration (m*M*)		Lactate concentration (m*M*)	
	0 h	216 h	0 h	216 h	0 h	216 h
def	54.9	25.5	2.6	20.8	0	17.6
afg	54.3	15.0	3.4	22.5	0	5.3
beg	54.6	33.4	3.4	18.9	0	16.8
abd	55.0	0	3.7	25.9	0	0
cdg	55.9	26.1	3.1	20.2	0	22.2
ace	56.6	4.3	3.3	17.9	0	0
bcf	57.0	36.3	2.8	16.2	0	17.1
abcdefg	56.3	49.9	5.2	19.0	0	0
Control (Insect-XPRESS)	34.0	0	4.0	27.6	0	0

[a] For hydrolysate designation *see* Table 1.

TABLE 3

EFFECTS OF HYDROLYSATES ON MAXIMAL CELL DENSITY

2^{7-4} fractional factorial experiment		Complete factorial experiment	
Hydrolysate	Effect	Factor	Effect
A	1.915	Yeastolate	1
B	−0.035	Primatone	0.3
C	0.165	Yeastolate–Primatone interaction	0.1
D	−0.515		
E	−0.815		
F	−1.065		
G	−0.585		

Cell 405, respectively. Thus, the YPR medium can support the growth of High-Five cells at high growth rates while, at the same time, avoiding the abrupt decrease in cell concentration and viability occurring in other media (Fig. 3*A*).

Sf-9 cells had quite distinct growth profiles under serum-free conditions (Fig. 3*B*). Indeed, growth in Insect-XPRESS and Sf-900 II media was characterized by high growth rates and short PDTs (0.0335 h^{-1} [20.7 h] and 0.0316 h^{-1} [21.9 h], respectively). On the other hand, in both YPR medium and HyQ SFX-Insect, the specific growth rates were considerably lower and the PDTs higher (0.0218 h^{-1} [31.8 h] and 0.0212 h^{-1} [32.7 h], respectively). In addition, a lag phase of about 2 d occurred for the YPR medium. This difference could be due to the fact that different cell batches have been used for growth studies (*see* "Materials and Methods"). In the case of the latter two media, we used Sf-9 cells which had been previously growing in serum-containing monolayer cultures. Despite the higher inoculation densities, cells could still need growth-promoting factors present in serum but absent from the serum-free formulations (Hensler et al., 1994). Nonetheless, Sf-9 cells in YPR medium reached the highest final density (5.4×10^6 cells/ml) compared to the other three media (Fig. 3*B*).

The potential of the YPR medium to support insect cell growth on a larger scale was tested in batch bioreactor cultures (Fig. 4*A* and *B*). The growth profiles of High-Five cells in 1-L batch cultures in Insect-XPRESS and YPR medium under identical culture conditions (DO = 50%, T = 27° C) are shown in Fig. 4*A*. The growth was slower in YPR medium ($\mu = 0.0267\ h^{-1}$) compared to Insect-XPRESS ($\mu = 0.036\ h^{-1}$) and the final viable cell concentration was somewhat lower (3.6×10^6–4.2×10^6 cells/ml). Cells in YPR medium maintained a prolonged stationary phase and a more gradual loss of viability, compared to Insect-XPRESS. Glucose specific uptake rates were comparable in the two media for the exponential growth phase, as can be seen in Table 4. Ammonia production rate,

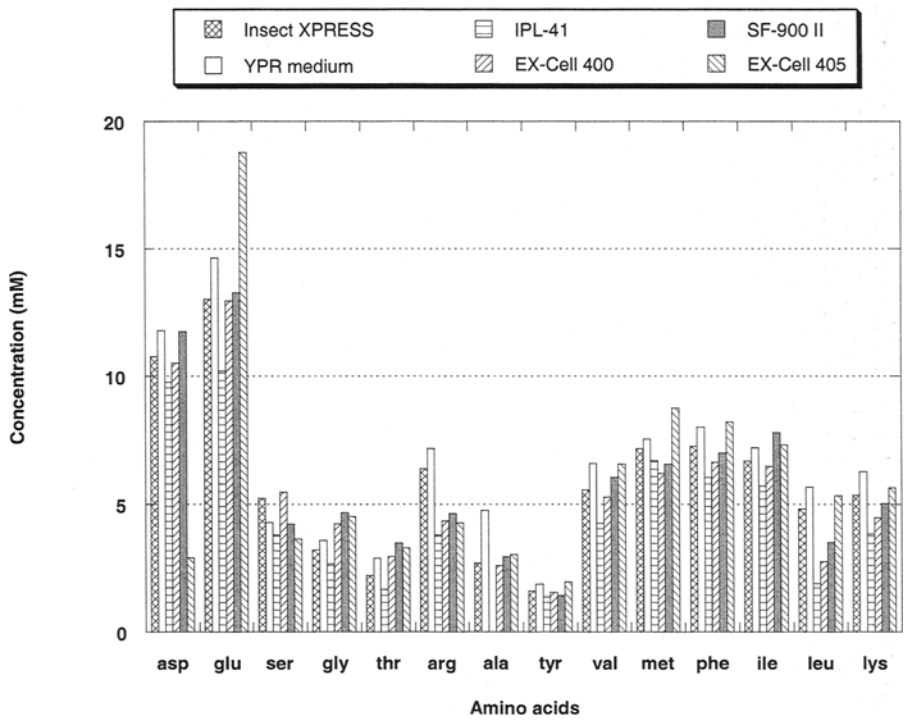
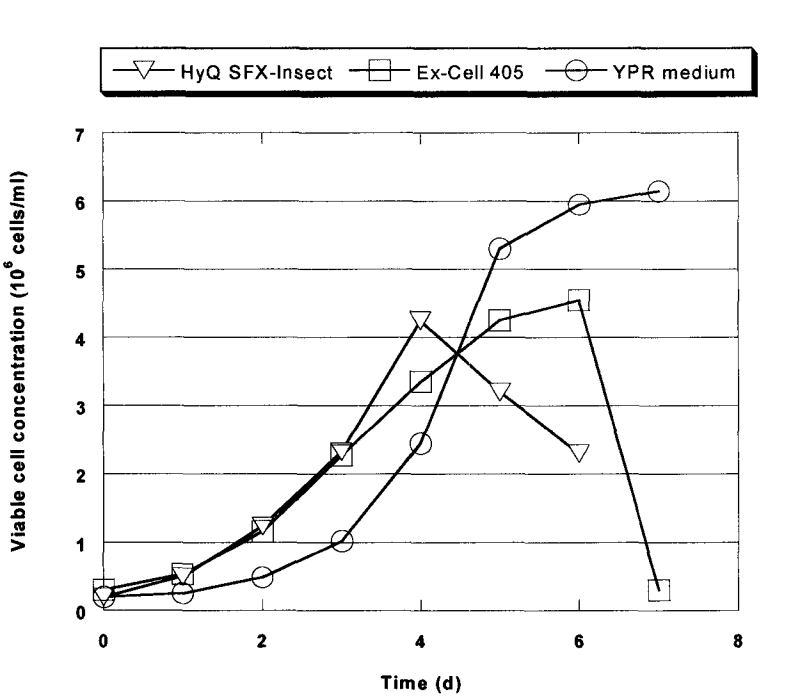


FIG. 2. Amino acid content of several serum-free media for insect cell culture. Amino acid concentrations were determined from fresh medium samples by HPLC except for the IPL-41 medium, the composition of which is given in Weiss et al. (1981).



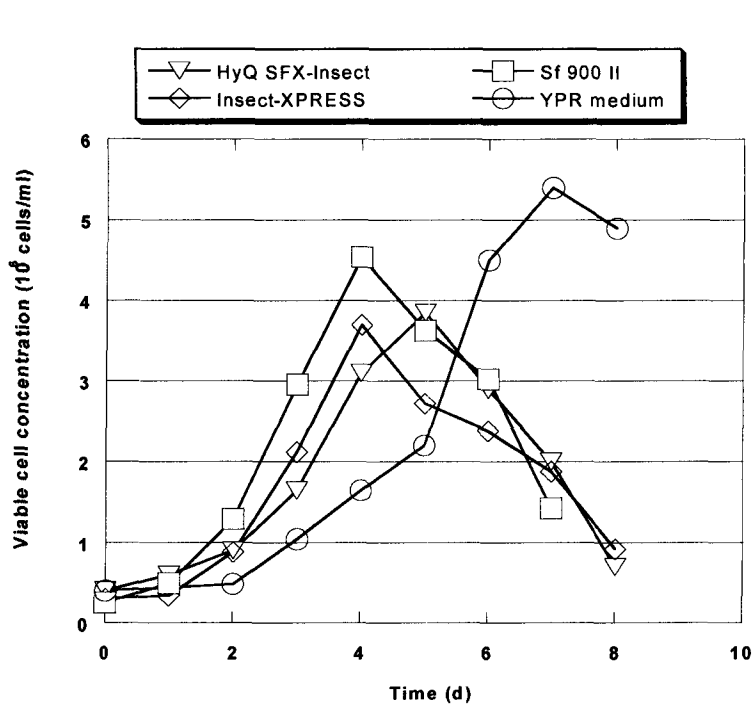


FIG. 3. Comparison of cell growth in several serum-free media. All data shown are from duplicate flasks: (*A*) High-Five cells; and (*B*) Sf-9 cells.

which reflects the consumption of amino acids, such as glutamine and asparagine was 1.5 times higher in Insect-XPRESS than in YPR medium. No lactate accumulation was observed in either medium, contrary to previously reported results (Rhiel et al., 1997).

Batch bioreactor cultures for Sf-9 cells were performed under the same conditions as for High-Five cells (Fig. 4*B*). The important difference in specific growth rates and consequently in PDT (Table 4) can, once again, be explained by the different origin of Sf-9 cells used in the bioreactor cultures. Sf-9 cells in YPR medium reached a higher final cell concentration (4.4×10^6 to 3.8×10^6 cells/ml) and maintained it for 3 d. Viability was also very high during both exponential and stationary phases, exceeding 95%. Cell concentration started to decline only when glucose was completely exhausted, which was also the case with High-Five cells (data not shown).

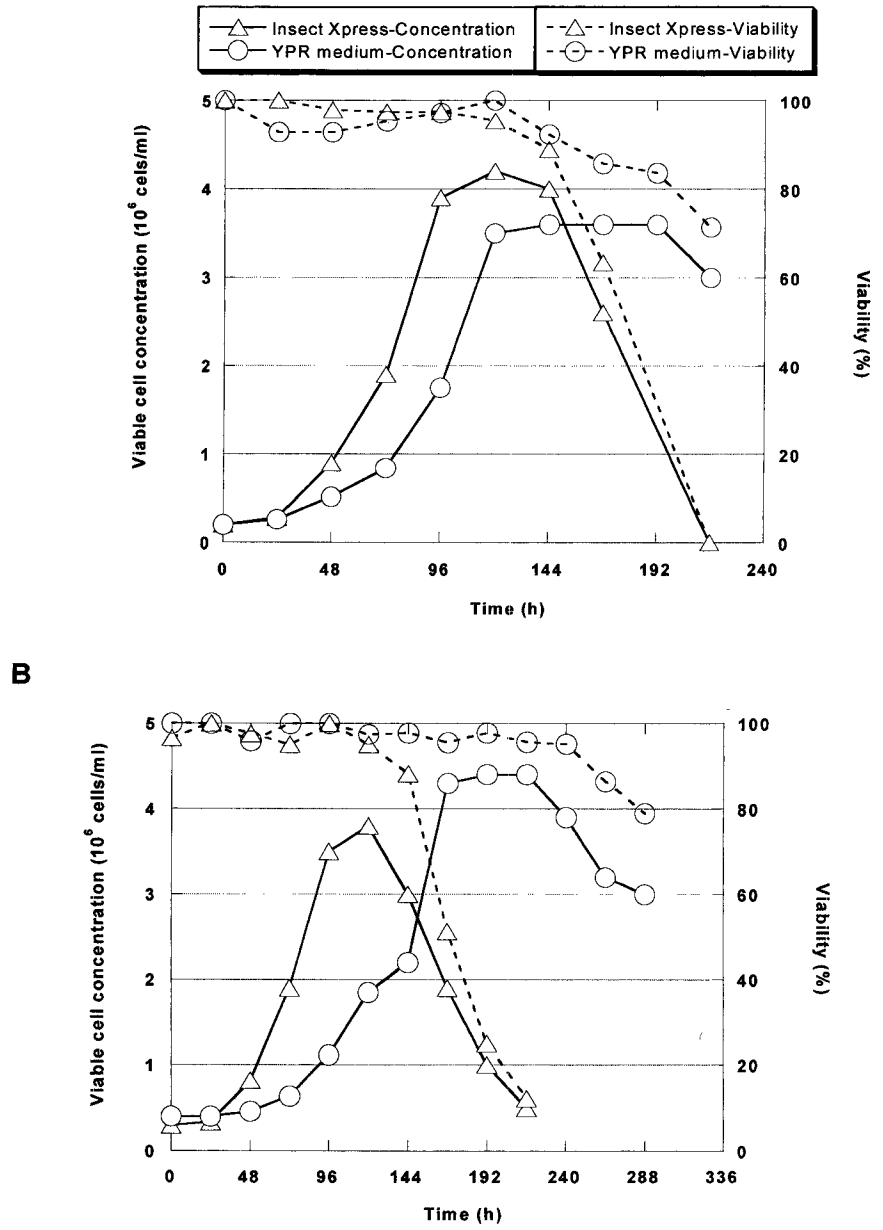


FIG. 4. Batch culture of insect cells in a Celliben Plus bioreactor (working volume 1 L) with two serum-free media: (*A*) High-Five cells; and (*B*) Sf-9 cells.

TABLE 4

METABOLIC AND KINETIC PARAMETERS FOR Sf-9 AND HIGH-FIVE CELLS IN BIOREACTOR BATCH CULTURES

	High-Five		Sf-9	
	YPR medium	Insect-XPRESS	YPR medium	Insect-XPRESS
Final cell density (10^6 cells/ml)	3.6	4.2	4.4	3.8
Specific growth rate (h^{-1})	0.0267	0.036	0.0183	0.0326
Population doubling time (h)	26	19.2	37.9	21.3
Glucose specific uptake rate (10^{-17} mol/cell × s)	−4.5	−5.2	−5.0	−2.6
Ammonia specific production rate (10^{-17} mol/cell × s)	2.6	4.1	0.5	

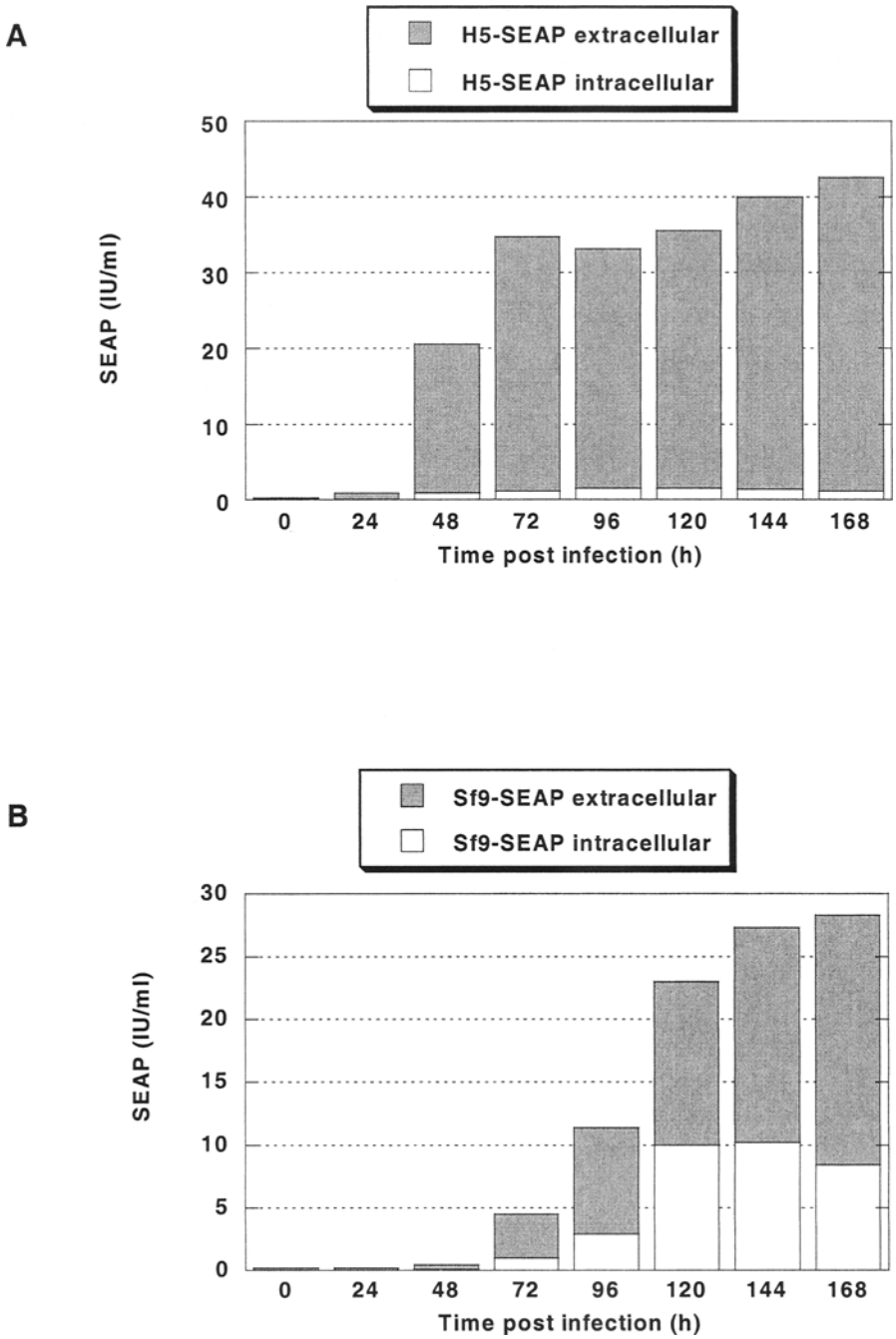


FIG. 5. Volumetric production of SEAP by insect cells in YPR medium. Infections were performed in duplicate flasks: (*A*) High-Five cells; and (*B*) Sf-9 cells.

There was no lactate production in either of the media and ammonia accumulation was minimal (data not shown). Surprisingly, glucose uptake rate in YPR medium was almost double than in Insect-XPRESS, at least for the exponential phase of growth (Table 4). Sf-9 cells in Insect-XPRESS were characterized by a rapid growth (PDT = 21.3 h) but their concentration and viability decreased abruptly soon after the maximal density had been reached.

Infection studies in YPR medium. The concentration and SEAP profiles for infected High-Five cells are shown in Fig. 5*A*. Cell growth continued for 72 h after infection, suggesting a nonsynchronous infection (Fig. 6). From 96 h on, cell concentration and viability declined rapidly as more and more cells lysed due to the infection. It should be noted that cells began to produce and efficiently secrete SEAP as early as 48 h after infection (Fig. 5*A*). In fact, at 48 h, total SEAP concentration was almost one-half of its final value. Final total SEAP titer reached 43 IU/ml (Fig. 5*A*).

The production profile of Sf-9 cells was quite different (Fig. 5*B*). These cells continued growing for 72 h after infection and SEAP production started at 72 h (Figs. 5*B* and 6). Since the initial conditions of infection density and MOI were the same for the two cell lines, this suggests that Sf-9 cells are less susceptible to infection by this AcMNPV construct than High-Five cells. SEAP secretion

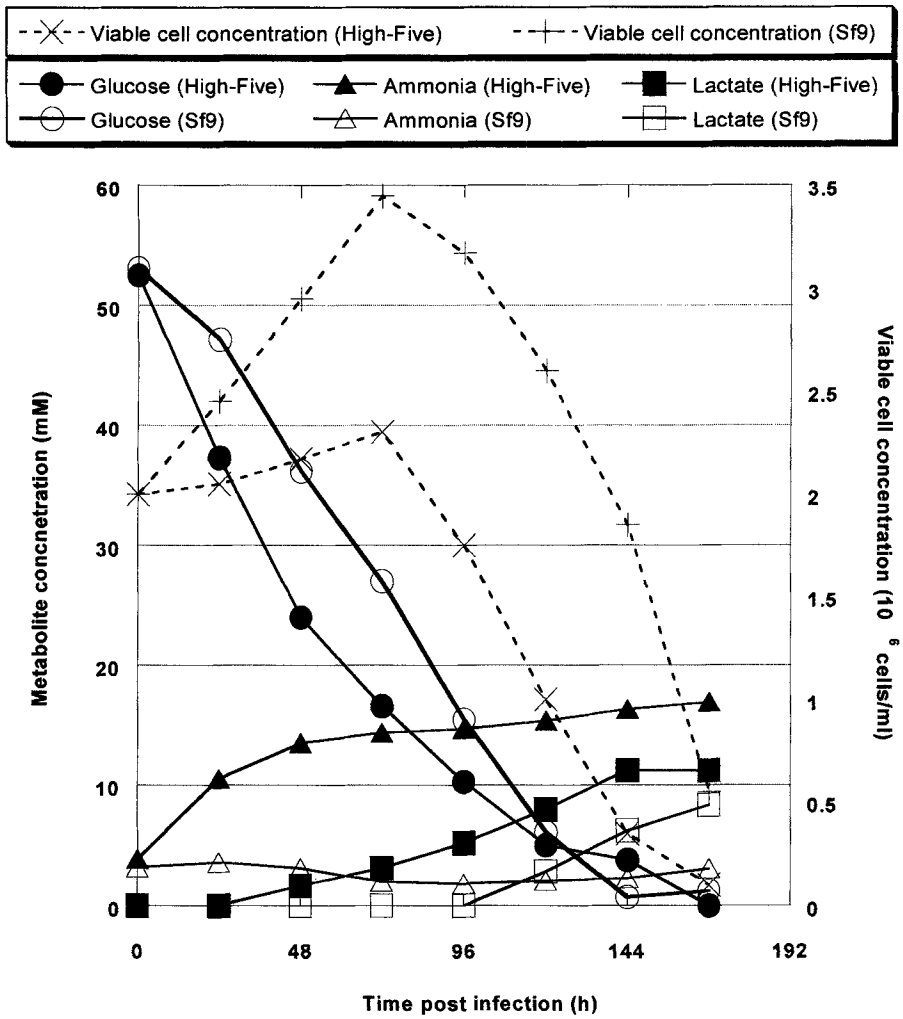


FIG. 6. Time course of metabolite concentration and viable cell concentration for High-Five and Sf-9 cell cultures in YPR medium infected with a SEAP-encoding baculovirus. Data shown are the average of duplicate flasks.

was not as efficient either (Fig. 5*B*). Even at 168 h after infection when viable cell concentration and viability were quite low, 30% of SEAP produced was intracellular. Final titer was lower (28 IU/ml) compared to High-Five cells (Fig. 5*A* and *B*).

Metabolite profiles were also quite distinct between cell lines during infection (Fig. 6). Glucose was nearly exhausted by 168 h after infection, but at that time viable cell concentrations were very low. Thus, protein production was not limited by glucose. High-Five cells consumed glucose rapidly during the first 48 h, but the slope of glucose consumption rate changed thereafter as infection progressed. Both cell lines accumulated lactate, starting at 24 h for High-Five and 96 h for Sf-9 cells (Fig. 6).

DISCUSSION

A fractional factorial experiment allowed us to screen a number of hydrolysates of various origins in the interest of developing a serum-free medium for the High-Five and Sf-9 cell lines. Yeastolate ultrafiltrate was found to have the most pronounced effect on cell growth. Its importance for insect cell growth and protein production by the baculovirus system has been noted by several authors (Maiorella et al., 1988; Nguyen et al., 1993; Bédard et al., 1994; Wu and Lee, 1998). Although it is a rich source of vitamin B complex (Vaughn and Fan, 1997), it seems that its growth-promoting effect is due to other substances (Wu and Lee, 1998). Yeastolate concentration up to 8 g/L led to high cell density for Sf-9 cell culture but further increase to 16 g/L inhibited growth (Drews et al., 1995). The complete factorial experiment we performed to determine the optimal concentrations of yeastolate and Primatone RL showed that an increase from 4 to 6 g/L in yeastolate concentration increased final cell concentration by 1.1×10^6 cells/ml (Fig. 1*B*). As yeastolate is quite expensive, i.e., it accounts for about 80% of the YPR medium cost, no further increase in its concentration was attempted.

The extended stationary phase observed for both bioreactor batch cultures and in several instances for shake-flask cultures was quite remarkable. In fact, in other serum-free media there was always an abrupt decrease in viability once the maximal cell concentration was attained. As most commercial media for insect cell culture contain yeastolate, it is not likely that the stationary phase is due to this ingredient. A significant prolongation of the stationary phase has been observed previously with mouse hybridoma culture, in which Primatone RL served as serum substitute and it was attributed to its antiapoptotic properties (Schlaeger, 1996b). In our case, the stationary phase was maintained as long as glucose was available. It thus appears that a high concentration of glucose, apart

from being essential for cell growth, can play a significant role in the delay of cell death when combined with Primatone RL in insect cell culture. Although the animal origin of Primatone can potentially be a source of problems, such as mycoplasma infection, variation of lot-to-lot and presence of impurities, it has been shown to be suitable for large scale mammalian and insect cell culture (Mizrahi, 1977; Schlaeger et al., 1993; Schlaeger, 1996b; Vaughn and Fan, 1997). Solutions to the above problems could be its better standardization by ultrafiltration (Schlaeger, 1996b) or the isolation of its fractions with growth-promoting and antiapoptotic properties (Amborski and Moskowitz, 1968). Especially in the case of High-Five cells, variations in quality from batch to batch can affect cell growth and final cell density. The use of this cell line for medium development proved to be entirely justified. Growth tests with rice, pea, and wheat hydrolysates from another source, which were characterized by a higher degree of hydrolysis than the Quest hydrolysates, resulted in high cell mortality within 24–48 h at concentrations of 0.3 and 0.5% (data not shown). Thus, judicious selection of hydrolysates seems to be an essential first step in medium development. High-Five cells showed different growth and metabolism profiles in different hydrolysate combinations. The lactate accumulation for some hydrolysate combinations does not seem to be due to oxygen limitation as the culture conditions rule out hypoxic conditions in the flasks (Neutra et al., 1992). Thus, a stress from a combined effect of two or more hydrolysates apparently led to decreased growth and lactate accumulation. There was an extended lag phase in the culture containing all of the hydrolysates as well as a low final cell concentration that can be attributed to either an inhibitory effect of this combination or to the high osmolarity of the medium. Furthermore, isolation of well-defined fractions of both yeastolate and Primatone RL could contribute to new media formulations as recently suggested for plant hydrolysate preparation (Franek et al., 2000).

Growth studies in shake-flasks and bioreactor batches showed clearly that YPR medium can support high densities for both Sf-9 and High-Five cells (Figs. 3 and 4). The relatively slow growth rates for Sf-9 cells can potentially be overcome by inoculation at higher densities (Hensler et al., 1994). The accumulation of by-products (ammonia, lactate) for Sf-9 cells was minimal, so YPR compares favorably in this aspect with other serum-free media. Although ammonia levels were not very high, a reduction of free amino acids and especially glutamine and asparagine could be desirable in the case of High-Five cell batch culture. We were unable to measure cystine consumption, which is reported to be essential for both Sf-9 and High-Five cells (Danner et al., 1995; Vaughn and Fan, 1997). Apart from asparagine and glutamine which were rapidly exhausted in bioreactor culture of High-Five cells in Insect XPRESS medium (data not shown) no other amino acid was consumed to any great extent in bioreactor cultures of Sf-9 and High-Five cells (Ikonomou et al., 1999). The consumption profiles can be different in the case of perfusion cultures and exhaustion of certain amino acids can occur. Therefore, YPR medium is also suitable for this mode of operation as it is characterized by a high concentration of all amino acids. For most of them, their level in the YPR medium is comparable to that of commercially available media (Fig. 2). Nevertheless, the free amino acids supplied from the hydrolysates account for 30% of the total free amino acids. Unless peptide-bound amino acids are efficiently taken up by the cells, it is quite doubtful that hydrolysates can be the only source of amino acids for cell growth (Vaughn and Fan, 1997).

YPR medium was shown to be quite effective for the production of secreted recombinant proteins, such as SEAP. Although a non-synchronous infection took place for both cell lines, and there was some cell growth after infection (Fig. 6), volumetric production of SEAP was quite high in the case of High-Five cells. The maximal value found in this work (43 IU/ml) compares favorably with values of ~50 IU/ml in ISYL and EX-Cell 405 media (Donaldson and Shuler, 1998) and ~41.5 IU/ml at 21% gas-phase O_2 (Dee et al., 1997). A much higher reported value of 82.4 IU/ml at 80% gas-phase O_2 (Dee et al., 1997) indicates the importance of oxygen-rich aeration during infection at least for SEAP production. The maximal total SEAP value for Sf-9 cells (28 IU/ml) compares well with the value of 29.1 IU/ml of the Sf21 cell line under increased oxygen level (80%) (Taticek and Shuler, 1997). The higher SEAP production in High-Five cells compared to Sf-9 cells and the superior secretion capacity of the former (over 96% of total SEAP was secreted from the beginning of production in our case) are in agreement with previous observations (Davis et al., 1993; Wickham and Nemerow, 1993; Saarinen et al., 1999).

Lactate accumulation occurred with both cell lines during infection. Although a transient increase in oxygen uptake rate (OUR) upon baculovirus infection is well established (Agathos, 1996), this does not seem to lead to oxygen limitation in our case. Lactate continued to accumulate until very late in the infection when typically OUR falls significantly, due to the viral cytopathic effect (Hensler and Agathos, 1994). Consequently, lactate accumulation during SEAP production should be attributed to stresses other than deficient oxygen supply. Ammonia level was relatively constant for the Sf-9 cell cultures but increased to 13 m*M* for High-Five cells during the first 48 h following infection (Fig. 6). Its slight concentration increase for the rest of the infection period suggests either an exhaustion of amino acids used as energy sources or their utilization for protein production.

The YPR medium appears to be an attractive alternative for the culture of Sf-9 and High-Five cells and the production of recombinant proteins. Its low cost of about 9.5€/L estimated on a 2000-L base, makes it especially suitable for industrial modes of operation, such as perfusion, which necessitate large medium volumes.

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