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Insect cell cultivation: growth and kinetics

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Introduction

The baculovirus-insect cell expression system has emerged as a fast and powerful tool for the production of numerous heterologous proteins which are a prerequisite e.g. for initiating random screening programs aimed at identifying low-molecular-mass non-proteinaceous drug substances, for performing X-ray crystallographic structure/function studies and rational drug design, and also for establishing "proof of principle" in animal studies of human diseases. If any of the above mentioned applications requires sufficiently large amounts of intact biologically active protein it will become necessary to carry out process optimization studies.

This review examines the progress made towards the cultivation of insect cells in controlled bioreactors with particular reference to the growth kinetics and protein expression under different physicochemical conditions and the published data on nutrient and by-product metabolic quotients during growth and infection. The focus is on the recent literature published until the end of 1994.

Suspension versus immobilized cultures

A limited number of publications over the past years dealt with the growth of insect cells in immobilized culture systems (Agathos *et al.*, 1990; Lazar *et al.*, 1987; Archambault *et al.*, 1994; Wickham & Nemerow, 1993; Kompier *et al.*, 1991; King *et al.*, 1989; Chung *et al.*, 1993). As the initial difficulties of cultivating insect cells in suspension using stirred tank or airlift bioreactors with submerged aeration or microsparging have been overcome (see below) and most insect cell lines can be adapted to grow in suspension, further research into such immobilized culture systems would only seem appropriate if strictly adherent cell lines showed greatly enhanced potential with respect to product yields and quality. Above all, the recovery of intracellular protein products may be difficult to achieve at large scale. In the following only suspension cultures will be considered.

Growth rates and maximal cell concentrations for suspension cells

Effect of different media and media supplements including serum

The kinetics of insect cell growth have by now been evaluated in quite an extensive number of investigations in controlled bioreactors. An overview of these is presented in Table 1. Most studies make use of insect cell lines derived from Spodoptera frugiperda, i.e., Sf9 or Sf21, and results are thus well comparable. Cell growth has been examined in airlift and stirred tank bioreactors with working volumes up to 150 l whilst maintaining oxygenation by bubble-free aeration, orifice sparging and microsparging or a combination of methods. Similar growth characteristics have been reported for cells cultivated in serum containing and serum-free media if shear protective agents like Pluronic F–68 are included in media formula-

tions. Optimal Pluronic F–68 concentration have been found to range from 0.1 to 0.3% (w/v) (Murhammer & Goochee, 1988; Zhang *et al.*, 1994; Caron *et al.*, 1990). The incorporation of undefined hydrolyzates like yeastolate, lactalbumin hydrolyzate or Primatone RL in the culture media has a marked influence on maximum cell densities. Limitations in any of the commonly quantitated medium components like carbohydrates, amino acids or lipids (Schmid, unpublished results) can be averted by increasing the initial concentrations or nutrient feeding over the course of the fermentation. Reported specific growth rates (μ) for Sf9 and Sf21 cells are in the range of 0.67–0.83 day^{-1}, which corresponds to average population doubling times of 20 to 25 hr. Maximum cell densities approach $10 \times 10E6$ viable cells ml^{-1} without additional feeding of nutrients (Schlaeger *et al.*, 1993). With feeding strategies (Nguyen *et al.*, 1993; Schlaeger *et al.*, 1992) or cell retention and medium perfusion (Caron *et al.*, 1994; Deutschmann & Jäger, 1994) biomass yields of up to $50 \times 10E6$ cells ml^{-1} have been obtained.

In most cases insect cell cultures are, however, infected with recombinant baculovirus preparations during the early part of the exponential growth phase and at viable cell concentrations well below the maximum cell densities that are indicated above. The challenge still remains to maintain a high level of protein expression (i.e., identical specific productivity), when infecting cultures at high cell density, and thus to increase the space-time-yield without complete medium exchanges before infection or continuous medium perfusion during infection as both of these approaches are difficult to perform in large scale operations.

Effect of hydrodynamic environment

Even in the recent literature there have been reported conflicting results with regard to the detrimental effects of sparging and microsparging on insect cell growth and culture viability. For example, Jain *et al.* (1991b) and Caron *et al.* (1990) reported identical growth characteristics for Sf9 cells using media supplemented with Pluronic F–68 as shear protectant regardless of whether surface aeration, orifice sparging, microsparging or bubble-free silicon tube gassing were employed for oxygenation purposes. On the other hand, Blanchard & Ferguson (1992) observed a negative effect of air sparging (compared to silicon tube gassing) on the viability of uninfected Sf9 cells using SF900 serum-free medium with 0.1% (w/v) Pluronic F–68. The extend of damage caused by submersed aeration or microsparging (if any) depends, among other things, on the hydrodynamics of the individual bioreactor, the (micro)sparger type and pore size, the gas flow-rate, and the type and concentration of added shear protectants. Many groups therefore now routinely use either stirred tank or airlift bioreactors at the pilot scale for insect cell cultivations. Guillaume *et al.* (1992) at Rhone-Poulenc Rorer found maximum cell densities and specific growth rates of Sf21 cells as well as the time-course of infection with 2 recombinant baculovirus constructs comparable for 2, 10 and 150 l stirred tank reactors when using pure oxygen sparging for DO control.

Recently, researchers at Merck (Junker *et al.*, 1994) reported on the use of a modified 75 l microbial fermenter for insect cell cultivations. At Hoffmann-La Roche we have used 25 and 75 l airlift as well as 150 l stirred tank reactors for the production of a variety of recombinant proteins from insect cells over the past years (Schlaeger *et al.*, 1992; Schlaeger *et al.*, 1995; Schmid *et al.*, 1994). Airlifts are operated at 0.03–0.07 VVM with additional microsparging at high cell densities. The 150 l vessels were conventional microbial bioreactors (Chemap) equipped with either a sail-type Teflon impeller or Rushton turbines. Oxygenation and pH control were achieved by orifice sparging of an air/oxygen/nitrogen/carbon dioxide mixture via a gas blending unit fitted with mass-flow controllers. Sf9 growth and expression of recombinant IFNγ receptors was identical to results obtained in airlift reactors (Schmid *et al.*, 1994 and unpublished results).

The effects of hydrodynamic forces on insect cells in suspension leading to increased cell damage or death are discussed in detail by Chalmers (see pp. 163–171, this volume).

Effect of dissolved oxygen concentration on growth characteristics

Despite a number of publications that report on the cultivation of insect cells under controlled dissolved oxygen (DO) conditions (e.g., Weiss *et al.*, 1989; King *et al.* 1992; Maiorella *et al.*, 1988; Caron *et al.*, 1990; Wong *et al.*, 1994; Kamen *et al.*, 1991; Scott *et al.*, 1992; Blanchard & Ferguson, 1992; Reuveny *et al.*, 1992 and 1993; Lazarte *et al.*, 1992; Nguyen *et al.*, 1993; Guillaume *et al.*, 1992) the effects of different DO concentrations on cell growth rates and maximum viable cell concentrations have only been evaluated in a limited number of bioreactor studies (Table 2).

Table 1. Growth characteristics of various insect cell lines in controlled bioreactors

Type of bioreactor	Working volume (l)	Cell line	Culture medium	Agitation/ Aeration	Physicochemical conditions	Specific growth rate μ (day^{-1})	Maximum cell counts (cells ml^{-1})	Reference
Airlift	21	S. frugiperda (Sf9)	IPL–41 with 10% FBS or serum-free	0.02 VVM	DO 20%	0.67–0.83	5.0 × 10E6	Maiorella *et al.*, 1988
STR, cell retention	1.4, 2, 6	S. frugiperda (Sf21)	IPL–41 with 5% FBS	rpm 40, bubble-free aeration	DO 40–100%	0.39–0.60	1.7–6.6 × 10E6 55 × 10E6 with perfusion	Deutschmann & Jäger, 1994
STR	4, 11	S. frugiperda (Sf9)	TNM-FH with 10% FBS IPL–41 serum-free	60–100 rpm, surface aeration	DO 30–40% no pH control	0.69–0.83 0.69–0.76	5 × 10E6 5.5 × 10E6	Caron *et al.*, 1990; Kamen *et al.*, 1991
STR, cell retention	4	S. frugiperda (Sf9)	TNM-FH with 10% FBS	rpm 70–80, microsparging	DO 30% no pH control	0.74 (batch), 0.36 (perfusion)	12–15 × 10E6 with perfusion	Caron *et al.*, 1994
STR	1.5	S. frugiperda (Sf9)	TC100 5% FBS IPL–41 5% FBS IPL–41 serum-free	rpm 50, bubble-free aeration	DO 40% pH 6.2	–	2.3 × 10E6 5.5 × 10E6 5.6 × 10E6	Fertig *et al.*, 1993
STR	2, 10, 150	S. frugiperda (Sf21)	EX-CELL 400 SFM	rpm 45, microsparging	DO 40% pH 6.2	0.65–0.79	5 × 10E6	Guillaume *et al.*, 1992
STR	2, 12	S. frugiperda (Sf9)	EX-CELL 400 SFM IPL–41 serum-free	rpm 200, 0.01 VVM, orifice sparging	DO 30% pH 6.3	0.83	3 × 10E6 12 × 10E6 with feeding	Nguyen *et al.*, 1993
Airlift	14	S. frugiperda (Sf9, Sf21)	SF–900 SFM	0.025–0.06 VVM	DO 10–20% no pH control	0.69	8 × 10E6	Wu *et al.*, 1992; King *et al.*, 1992
STR	1.5	S. frugiperda (Sf9)	IPL–41 with 5% FBS SF900II SFM	rpm 120, sparging	DO 30% pH 6.2	0.48 0.67	2–5 × 10E6 5–6 × 10E6	Power *et al.*, 1992; Power *et al.*, 1994
Airlift	10	S. frugiperda (Sf9, Sf21)	EX-CELL 400 SFM	0.02–0.04 VVM	DO 10–20% pH 6.2	0.83	2.9 × 10E6	Weiss *et al.*, 1989
Airlift	23	T. ni (BTI-Tn–5B1–4)	SF–1 with 1% FBS or serum-free	0.05 VVM and microsparging	DO 30% pH 6.1–6.3	0.82	4.5–6 × 10E6 8 × 10E6 with feeding	Schlaeger *et al.*, 1995
Airlift STR	23, 60 120	S. frugiperda (Sf9, Sf21)	IP301 or SF–1 with 1% FBS or serum-free	0.05 VVM, microsparging rpm 25–40, 0.02 VVM, ring sparger	DO 30–60% pH 6.1–6.3 DO 30% no pH control	0.67–0.76	9–13 × 10E6 depending on feeding strategy	Schlaeger *et al.*, 1993; Schlaeger & Schmid, unpublished results
STR Airlift	1.5 0.6	S. frugiperda (Sf9)	TNM-FH with 5% FBS	rpm 200, sparging 0.005 VVM	DO 50% no pH control	0.69–0.88 0.78–0.89	2–2.5 × 10E6	Murhammer & Goochee, 1988
STR	5	S. frugiperda (Sf9)	IPL–41 with 10% FBS ICSF-WB SFM	rpm 80, orifice sparging	DO 65% pH 6.2	0.67–0.88 0.69–0.88	4–5 × 10E6 4–5 × 10E6	Reuveny *et al.*, 1992
STR	8, 18	S. frugiperda (Sf9)	IPL–41 with 2% FBS	bubble-free aeration or microsparging	DO 10–110%	0.48–0.65	4–5 × 10E6	Jain *et al.*, 1991a, b
STR	1.5	B. mori (Bm5)	IPL–41 with 10% FBS	rpm 60–85, sparging	DO 10–60% pH 6.3	0.46	3.6–4.8 × 10E6	Zhang *et al.*, 1994; Zhang *et al.*, 1993
STR	100	S. frugiperda (Sf9)	IPL–41 with 10% FBS	rpm 35, microsparging	DO 30% no pH control	0.69–0.83	1.5 × 10E6	Barkhem *et al.*, 1992
STR	1	S. frugiperda (Sf9)	SF900II SFM	rpm 140, surface aeration	DO 50% no pH control	0.74	7 × 10E6	Wong *et al.*, 1994

VVM = volumes of gas per culture volume and minute;
STR = stirred tank reactor;
SFM = serum-free medium.

Table 2. Bioreactor studies examining the effect of different dissolved oxygen concentrations on the growth kinetics of insect cells

Cell line	DO setpoint (% air saturation)[a]	Type and size of bioreactor	Reference
Sf21	40, 70, 100	1.2 l stirred tank, bubble-free aeration	Deutschmann & Jäger, 1994
TN-368	15, 50, 100, >100	2–3 l stirred tank, sparging	Hink & Strauss, 1980; Hink, 1982
Sf9	10, 65, 110	18 l stirred tank, microsparging	Jain *et al.*, 1991a and 1991b
Sf9	20, 40, 60, 80	1.5 l stirred tank, bubble-free aeration	Klöppinger *et al.*, 1990
Sf9	30–60	23 and 60 l airlift, 120 l stirred tank, orifice sparging	Schlaeger & Schmid, unpublished results
Sf9	5, 10, 50, 100	0.25 l stirred tank, surface aeration	Hensler & Agathos, 1994
Bm5	10, 20, 30, 40, 60	1.5 l stirred tank, CelliGen	Zhang *et al.*, 1994

[a] Optimal values that gave highest specific growth rates and cell densities are underlined.

In early studies Hink & Strauss (1980) and Hink (1982) examined the growth characteristics of the Trichoplusia ni TN–368 cell line in sparged stirred tank reactors with working volumes of 2 to 3 l. The specific cell growth rates were found to be similar at all DO levels (maximum growth rate 1.19 day^{-1}). However, cells cultivated at 15% DO were vacuolated at 120 hr, this being followed by a rapid decrease in cell numbers. Cells maintained at DO >100% exhibited lower maximum cell densities. Klöppinger *et al.* (1990) investigated the effects of different DO setpoints (20, 40, 60 and 80%) on Sf9 cell growth in batch cultures using a 1.5 l stirred tank bioreactor with bubble-free aeration. Maximum specific growth rates ($\mu = 0.76\ day^{-1}$) were measured for DO levels of 20 and 40%. At 60 and 80% DO these growth rates were only reduced by ~10%. Using TC100 medium supplemented with 10% FBS a maximum viable cell concentration of 2.3 × 10E6 cells ml^{-1} was observed at 40% DO in this series of experiments. Similar experiments were also performed by Jain *et al.* (1991a, b) in an 18 l stirred tank reactor equipped with 2 μm pore size microspargers. The levels of DO in the culture medium had a significant effect on the growth rate of cells. At 10 and 110% DO the specific growth rates were ca. 25% lower than at 65% DO ($\mu = 0.65\ day^{-1}$). The authors speculated that at low DO cells probably were oxygen-starved and at high DO were experiencing oxygen toxicity effects. Under all three conditions no differences were found for cell viabilities (ca. 98%). This seems to imply that the reduced growth rates of cells are not the result of increased cell death but a direct consequence of the DO concentration in the culture medium. At Hoffmann-La Roche we have consistently observed maximum specific growth rates of $0.73 \pm 0.03\ day^{-1}$ for Sf9 cells cultivated over a range of dissolved oxygen concentrations (30 to 60% DO) in airlift and stirred tank bioreactors (Schlaeger & Schmid, unpublished results). Both, low-serum containing (1% FBS) or protein-free media IP301 and SF–1 (Schlaeger *et al.*, 1993) supplemented with lipids (in the form of fatty acid/sterol containing microemulsions or lipoprotein fractions) and Pluronic F–68 support cell growth to final densities of ca. 1 × 10E7 cells ml^{-1} without nutrient feeding. Hensler & Agathos (1994) found, contrary to the results obtained by Jain *et al.* (1991a, b), that Sf9 cells showed no difference with respect to specific cell growth rates, maximum cell densities (5 × 10E6 cells ml^{-1}) and cell viabilities, when cultivated in 0.25 l stirred tank reactors using surface aeration over the whole range of DO levels from 5 to 100%.

Studies on the influence of dissolved oxygen on the growth of 2 different insect cell lines have recently been published. Deutschmann & Jäger (1994) reported optimal growth of Sf21 cells (the parental line to Sf9) at 70% DO using a 1.2 l bioreactor equipped with a double-membrane stirrer for bubble-free aeration and medium perfusion. At 100% DO and unexpectedly also at 40% DO specific growth rates and maximum viable cell concentrations were adversely effected in batch experiments. At 40% air saturation maximum cell numbers were reduced more than threefold and population doubling times were increased ca. 50% compared to optimal conditions. Bombyx mori (Bm5) cell growth was evaluated by Zhang *et al.* (1994). Specific growth rates ($\mu = 0.46\ day^{-1}$) and maximum cell densities (ca. 4.3 × 10E6 cells ml^{-1}) were unaffected at DO levels between 20 and 60% air saturation. However, the maximum cell concentration was reduced to 3.6 × 10E6 cells ml^{-1} at 10% DO, which – as reasoned by Jain *et al.* (1991b) – could be due to the limited availability of oxygen for cellular functions.

Table 3. Bioreactor studies examining the effect of different dissolved oxygen concentrations on the product formation in insect cells

Cell line	DO setpoint (% air saturation)	Product expressed	References
Sf9	20, 40, 60, 80	polyhedra	Klöppinger *et al.*, 1990
Sf9	35, bioreactor (oxygen limitation)	epoxide hydrolase	Wang *et al.*, 1993
Sf9	10, 65, 110	half-antistasin (anticoagulant protein, inhibitor of Factor Xa)	Jain *et al.*, 1991a, b
Sf9	15, shake flask (oxygen excess)	β-galactosidase	Reuveny *et al.*, 1993
Sf9	10, 30, 50, spinner flask (oxygen limitation)	extracellular domain of human IFNγ receptor	Schmid *et al.*, 1994
Sf9	5, 10, 50, 100	β-galactosidase	Hensler & Agathos, 1994
BTI-Tn-5B1-4	15, 50	soluble human TNF p55 receptor	Schlaeger *et al.*, unpublished results
Sf9	5, 10, 50, 80, spinner flask (oxygen excess or limitation)	fusion protein of viral origin (accumulates in nuclei of infected cells)	Blanchard & Ferguson, 1992; Scott *et al.*, 1992
Bm5	30, 40	bacterial chloramphenicol acetyltransferase	Zhang *et al.*, 1994

Bm5 cells seems to be similar to Sf9 cells with regard to the observed broad optimum in dissolved oxygen concentration.

Although obtained with different culture media and bioreactor configurations, the sum of the above results seems to indicate that at least Sf9 and Bm5 cells can be grown over a wide range of DO concentrations extending from 20 to 65% DO at maximum specific growth rates and high cell densities. More extreme values in dissolved oxygen concentrations led to significantly reduced growth rates and cell concentrations except for the study communicated by Hensler & Agathos (1994).

Effect of dissolved oxygen concentration on recombinant protein and baculovirus production

Some groups also reported on the influence of different DO concentrations during the infection phase with wild-type or recombinant baculoviruses. Data eluding to the cell line under investigation, the dissolved oxygen during infection and the expressed protein are summarized in Table 3.

The effect of dissolved oxygen levels on the production of a truncated form of the anticoagulant and antimetastatic agent antistasin (H-ANS) was assessed in a silicon-tube gassed 8 l bioreactor to exclude any potential effects of sparging (Jain *et al.*, 1991a, b). Cells were grown at 65% DO and then infected at different DO concentrations. As observed for Sf9 cell growth (see above), it was found that infection of the culture at 65% DO gave optimal H-ANS values, whereas DO levels of 10 and 110% resulted in decreased product yields and an almost 2-fold reduction of the specific productivity. H-ANS concentrations reached their maximum values at 80 hr post-infection (65% DO) and subsequently decreased, as measured by a Factor Xa inhibition assay.

In my group we performed similar experiments to evaluate the effects of different DO levels during infection on the expression of full length extracellular domains of human and mouse IFNγ receptors (Schmid *et al.*, 1994). In one experiment we used 2 identical 25 l airlift bioreactors operated at a constant gas sparging rate of 0.05 VVM and a DO level of 50% during the growth phase of Sf9 cells. After identical batch growth, cultures were infected with the human recombinant virus preparation at a multiplicity of infection (MOI) of 1 pfu cells^{-1}. For one of the reactors the DO level was reduced to 10% air saturation ca. 15 min before infection. Data from ELISA determinations, functional binding assays, and electrophoretic analyses demonstrated a threefold increase in human IFNγ receptor concentrations when the infection process was carried out at the 10% compared to the 50% DO level. Multiple glycoforms of human (and mouse) soluble receptor(s) with apparent molar masses of 28 000 to 32 000 g mol^{-1} were observed for fermentation samples analyzed by SDS-PAGE under nonreducing conditions with subsequent ligand blotting or protein-staining. The pattern of identified isoforms varied as a function of infection time and DO level. Protein het-

erogeneity could be associated with either the unequal utilization of the five potential N-glycosylation sites or the linkage of different carbohydrate moieties to these sites or both (Fountoulakis *et al.*, 1991; Manneberg *et al.*, 1994). Taken together our data for the expression of human IFNγ receptor in Sf9 cells indicate that the highest expression levels (ca. 10 μg ml^{-1} at 5 days post-infection) are obtained at 10 and 30% DO, whereas at 50% DO and at potentially oxygen limiting conditions (simulated by transferring cultures from bioreactors directly after infection into spinner flasks with various ratios of culture to vessel volume) titers are drastically reduced. At day 6 (and later) of the post-infection period, ELISA and binding assay data indicate decreased concentrations of functionally active receptor protein, which may be a consequence of limited proteolytic degradation, changes in glycosylation pattern and/or other unidentified modifications.

Blanchard & Ferguson (1992) investigated the expression of a fusion protein of viral origin which accumulates in the nucleus of infected cells in a 3.5 l stirred tank bioreactor using EX-CELL 401 serum-free medium. Sf9 cells were cultivated at 50% DO and at a viable cell density of 2.3 × 10E6 cells ml^{-1} infected with recombinant baculovirus at an MOI of 1 pfu cell^{-1}. In this study the DO level was then simultaneously with the virus addition reset to maintain the levels for the post-infection period at either 80, 50, 10 or 5% DO. The highest product concentrations were determined at 50% DO during infection (corresponds to 82% of the titer measured for the reference flask under oxygen excess). At 10 and 80% air saturation protein yields were reduced by 18 and 50%, respectively. At the lowest DO level only 5% of the maximum product concentration was obtained. It should be noted that the above values indicate protein titers after an infection period of 44 hr; no time-course data is presented. Using a recombinant baculovirus encoding for the same or a similar protein of interest, Scott *et al.* (1992) found no expression in an oxygen-limited spinner flask and a maximum product concentration ca. 50 hr post-infection in the bioreactor (50% DO). The group observed a decrease in cell viablity from greater 90 to 50% after 2 days of infection for the culture maintained at 50% DO, which resulted in the highest product titers. This is in agreement with results by Schmid *et al.* (1994), who determined a lower remaining cell viability after 6 days of infection in the case of low (15%) dissolved oxygen concentration but higher IFNγ receptor concentrations. Jain *et al.* (1991b), however, did not observe an increased cell viability for 10% DO post-infection (compared to 65%) which was associated with 50% reduced H-ANS titers. Culture viability as well as cell volume (Schmid *et al.*, 1994; Jain *et al.*, 1991b) may be a useful indicator to follow during the infection period for a given project. However, a comparison of results from several groups is complicated by the use of various bioreactor configurations resulting in different hydrodynamic environments during infection and by the use of dissimilar recombinant baculoviruses for infection (expression vectors and protein product itself).

Wang *et al.* (1993) found the expression of epoxide hydrolase from Sf9 cells increased by 200% when the dissolved oxygen was maintained at ca. 35% DO during the infection period compared to the oxygen-limited control. Reuveny *et al.* (1993) presented data on the effect of DO levels on recombinant β-galactosidase production. Sf9 cells were propagated in a 5 l stirred tank reactor at 65% DO using orifice sparging. After 4 days of infection cultures maintained at 15% air saturation in the bioreactor yielded only 70% of product compared with cultures which were kept under conditions where oxygen was supposedly not limiting, i.e., shake flask cultures. However, when examining the time-course data for total β-galactosidase expression it seems as if the maximum concentration had been reached at day 4 post-infection in the shake flask culture, whereas titers in the bioreactor were still increasing significantly from day 3 to day 4. This may indicate that maximum β-galactosidase concentrations were not yet achieved in this case and stresses the importance of evaluating the complete time-course of protein expression during the post-infection period.

Contrary to the above studies and similar to their experiments that investigated the effects of DO on Sf9 cell growth, Hensler & Agathos (1994) observed expression of β-galactosidase at identical levels over a wide range of dissolved oxygen concentrations between 5 and 100% air saturation.

For bacterial chloramphenicol acetyltransferase (CAT) production in Bombyx mori (Bm5) cells Zhang *et al.* (1994) found no difference in CAT yields for infection at either 30 or 40% DO. Trichoplusia ni (BTI-Tn–5B1–4) cells were grown at 30% DO in 2 identical airlift bioreactors up to an infection cell density of 2.5 × 10E6 cells ml^{-1} by Schlaeger *et al.* (unpublished results). When the dissolved oxygen concentration was either adjusted to 15 or 50% air saturation during the infection period, product concentrations for soluble human TNF receptor p55 protein were determined to

be reduced by ca. 20% at the low DO level, whereby titers in the supernatant followed parallel time-courses. In a study that examined virus production in Sf9 cells at day 4 after infection with wild-type Autographa californica nuclear polyhedrosis virus, Klöppinger *et al.* (1990) reported that a dissolved oxygen concentration of 20% during infection reduced the yield of polyhedra per cell by more than 50% compared an oxygen concentration of 40 to 80%.

In summary, in all but one (Hensler & Agathos, 1994) of the investigations into the effect of DO levels on recombinant protein or polyhedra production significant differences in product yield were determined. From most publications it is not clear at what time exactly the dissolved oxygen level was changed from its growth phase value to the various post-infection values. It may be interesting to study the effect of DO more thoroughly, i.e., to adjust it to the desired levels some time before the addition of the baculovirus preparation, simultaneously with the virus addition, at the time of viral replication (15–24 hr post-infection) or later during the protein production phase. In any event, it is necessary to evaluate the complete time-course of product formation with respect to protein concentration and quality. Product quality is at least as important as total concentration because in the end it is the amount of intact biologically active product that determines the overall yield and productivity of any production process.

Effect of other physicochemical conditions

Temperature. Most insect cells can be cultivated over a temperature range of 25–30 °C (Agathos *et al.*, 1990); however, the optimal temperature during cell growth and infection for Sf9 cells is traditionally considered to be around 27–28 °C. In spinner flask studies Hild *et al.* (1992) achieved maximum cell densities and specific growth rates of 2.9–3.8 × 10E6 cells ml^{-1} and 0.67 day^{-1}, respectively, for Sf9 cells cultivated in TC100 medium with 5% FBS over a temperature range of 26–30 °C. Reuveny *et al.* (1993) found a temperature of 27 °C optimal for the growth of Sf9 cells resulting in the highest maximum cell concentrations and a specific growth rate of 0.67 day^{-1}. Already at 25 °C the specific growth rate was reduced by 30%. At 30 °C (while the specific growth actually was increased) an immediate and dramatic decrease in cell viability was observed after the maximum cell density was reached. This study appears to be the only one published where the effects of temperature on recombinant protein expression were examined. Exponentially growing cells cultivated under controlled conditions in 5 l bioreactors (27 °C) were resuspended in fresh medium at 0.5 × 10E6 cells ml^{-1} and incubated in spinner flasks at different temperatures. Cells were infected at MOI of 3 with recombinant baculoviruses and the expression of β-galactosidase and human glucocerebrosidase was monitored both in the cell pellet and in the supernatant. The total expression levels at 27 °C were similar to those obtained at 22 and 25 °C; lower yields were obtained at 30 °C. An increase in temperature from 22 to 27 °C led to an earlier infection of cells, as indicated by earlier expression of proteins, and to an increase in the proportion of both products released into the medium. No analyses of protein quality (e.g., degradation or glycosylation) were performed. **pH-value.** Medium pH-values required for optimal in vitro growth of various insect cells range between pH 6 and 7 as given in the literature (Sohi, 1980; Hink, 1982; Kurtti & Munderloh, 1984). Hild *et al.* (1992) found maximum cell densities and specific growth rates of 3.1–3.8 × 10E6 cells ml^{-1} and 0.72 day^{-1}, respectively, for Sf9 growth over a range of pH-values between 6.2 and 6.4. In a recent study Zhang *et al.* (1994) reported on the effect of pH on cell growth for Bm5 cells in 1.5 l bioreactors at a controlled DO of 40% air saturation. The highest specific growth rates (0.43–0.46 day^{-1}) and maximum cell densities (approaching 4.5 × 10E6 cells ml^{-1}) were obtained in the pH range from 6.1 to 6.3. At lower and higher pH-values increased lag times, reduced specific growth rates, and decreased maximum viable cell densities were observed. Similar optimal values of pH 6.0 to 6.25 and pH 6.2 to 6.8 were reported by Hink & Strauss (1980) for Trichoplusia ni (TN–368) cells and by Sohi (1980) for three lepidopteran cell lines, respectively. A pH-value of 6.2 is generally used for Sf9 cell growth in controlled bioreactors. The influence of medium pH-values on recombinant protein expression has possibly never been thoroughly investigated. Zhang *et al.* (1994, 1993) noted that recombinant chloramphenicol acetyltransferase (CAT) production in Bm5 cells was reduced by >50%, when the pH-value during infection was controlled at pH 6.5 instead of pH 6.3 (STR, 80 rpm, DO 40%, 28 °C). **Osmolality.** The same authors reported for Bombyx mori (Bm5) cells in flask experiments a maximum cell density at a medium osmolality of about 370 mosm kg^{-1}. Greater than 90% of the maximum cell density was achieved with a medium osmolality between 350 and 385 mosm kg^{-1}. In earlier studies reported values of optimal medium osmolalities for the growth of var-

ious insect cell lines vary between 250 and 450 mosm kg^{-1} (Sohi, 1980; Kurtti & Munderloh, 1984; Kurtti *et al.*, 1974; Kurtti *et al.*, 1975). Typical insect cell culture media are adjusted to an initial osmolality of 330–375 mosm kg^{-1} (Weiss *et al.*, 1981 and 1989; Schlaeger *et al.*, 1993; Wilkie *et al.*, 1980; Inlow *et al.*, 1989), whereas culture media for mammalian cells (hybridomas, CHO) usually have an osmolality of 280–320 mosm kg^{-1}. No studies have been reported on the influence of culture osmolality during the infection phase of insect cells.

Metabolic studies in batch and continuous cultures

Substrate and by-product metabolic quotients

Data on metabolic quotients for insect cells in the published literature is inconsistent. Hensler and Agathos (1994) determined that glucose and glutamine were consumed at 60–70% higher rates 24 hr after infection, at which time a maximum value in specific oxygen uptake rate was observed (see below). The specific glucose (qGluc) and glutamine (qGln) consumption rates were increased from 1.1 and 0.9 mmol/10E9 cells × d for uninfected cells to 1.8 and 1.5 mmol/10E9 cells × d for β-galactosidase-infected cells. Zhang *et al.* (1993) also found glucose uptake rates increased from 1.3 to 2.0 mmol/10E9 cells × d after viral infection, whereas Reuveny *et al.* (1992) found qGluc during the first 2 days after infection at same level or even reduced for either serum-containing or serum-free medium. Recent data by Wong *et al.* (1994) is in agreement with the latter results. Reductions in both qGluc and qGln were observed after infection with recombinant baculovirus, although a 30% increase in specific oxygen uptake rates was noted. The authors found some amino acid consumption rates (asparagine, arginine, glycine, threonine) elevated, but no indication that glucose or glutamine were responsible for the increased qO_2 after infection. They speculated that lipid catabolism is possibly contributing to the energy supply post-infection. Differences in nutrient consumption and by-product formation rates were observed by Reuveny *et al.* (1992), Kamen *et al.* (1991) and Bédard *et al.* (1993) as a function of culture medium. For a serum-free culture medium that contained glucose as the sole carbohydrate, Reuveny *et al.* (1992) calculated a 100% increase in qGluc compared to standard IPL–41 medium with 10% FBS that contained sucrose, maltose, glucose. Deutschmann & Jäger (1994) found the highest specific glucose uptake rate for Sf21 cells at 70% DO (1.5 mmol/10E9 cells × d). Under these conditions they observed the best growth characteristics with respect to specific growth rate and cell density. Uptake rates were reduced at lower specific growth rates (40 and 110% DO) to 0.8 and to 0.4 mmol/10E9 cells × d, respectively. No lactate formation was noted at 70 and 110% air saturation, however, the specific lactate formation rate was estimated at 3.6 mmol/10E9 cells × d at 40% DO. Schlaeger *et al.* (1995) compared qGluc and qGln during the exponential growth phase for Sf9 and BTI-Tn–5B1–4 cells cultivated in SF–1 medium. Specific rates for both nutrients were found to be higher for T. ni cells (qGluc 2.5 and qGln 1.1 mmol/10E9 cells × d) than for Sf9 cells (qGluc 1.9 and qGLN 0.6 mmol/10E9 cells × d).

Oxygen consumption rates

Volumetric oxygen consumption rates serve as one of the key design parameters for insect cell baculovirus production processes as they do for any other aerobic fermentation process. Over the past years several researchers and groups have reported specific oxygen uptake rates (qO_2) for insect cells during growth and subsequent infection with wild-type and recombinant baculoviruses (Table 4). From these values the volumetric oxygen demand can be estimated. The demand for insect cells may reach values as high as 100–150 mmol l^{-1} × d. This compares to typical oxygen uptake rates of ca. 70 mmol l^{-1} × d for plant cells (Fowler, 1987) and of 100–2000 mmol l^{-1} × d for microorganisms (Enfors & Mattiasson, 1983).

Specific oxygen consumption rates determined for insect cells are similar to those obtained for mammalian cells (Spier & Griffiths, 1984; Fleischaker & Sinskey, 1981; Aunins & Henzler, 1993). Most research groups report an increasing specific oxygen requirement after infection with baculovirus preparations. The extent of increase varies significantly for the different studies. Variations may be due to the use of wild-type or recombinant baculoviruses, the differences in expression vectors and protein product, the multiplicity of infection (fraction of defective virus particles), the exact physiological state of cells at the time of infection or the physiological conditions during the infection period. Qualitatively the phenomenom of higher respiratory activity is attributed to increased metabolic rates (see above) that result from viral replication and virus-induced macromolecule biosynthesis.

Table 4. Specific oxygen consumption rates for different insect cells during growth and infection

Cell type	Cell line	Specific oxygen consumption rate (mmol/10E9 cells × d)		References
		Growth	Infection	
Insect	T. ni (TN–368)	8.6	17.3	Streett & Hink, 1978
	S. frugiperda (Sf9)	3.7 (exp. phase)	4.1	Maiorella *et al.*, 1988
	S. frugiperda (Sf9)	2.0–7.8 as function of DO	1.7–8.5	Jain *et al.*, 1991b
	S. frugiperda (Sf9)	9.2–11.4 (exp. phase)	9.5–3.7	Schmid *et al.*, 1994
	T. ni (BTI-Tn–5B1–4)	6.5–7.6 (exp. phase)	7.8–7.3	Schlaeger *et al.*, unpublished results
	S. frugiperda (Sf9)	4.8–6.5	7.7	Kamen *et al.*, 1991
	S. frugiperda (Sf9)	3.4–5.2	7.4–3.9	Schopf *et al.*, 1990
	S. frugiperda (Sf9)	4.8	–	Archambault *et al.*, 1994
	S. frugiperda (Sf9)	7.0	9.6	Scott *et al.*, 1992
	S. frugiperda (Sf9)	9.0–11.3 serum-free 5.3 serum-containing	–	Reuveny *et al.*, 1992 and 1993
	S. frugiperda (Sf21)	0.7–3.4 as function of DO	–	Deutschmann & Jäger, 1994
	S. frugiperda (Sf9)	4.7 (exp. phase) 1.9 (stat. phase)	3.9–2.7	King *et al.*, 1992
	S. frugiperda (Sf9)	5.3 (exp. phase)	6.9–4.0	Wong *et al.*, 1994
	S. frugiperda (Sf9)	7.9–8.4 serum-free 5.3 serum-containing	14.4–2.4	Hensler & Agathos, 1994; Hensler *et al.*, 1994
Mammalian	Various human cells, murine myelomas and hybridomas, recombinant CHO cell lines	0.8–13		Spier & Griffiths, 1984; Fleischaker & Sinskey, 1981; Aunins & Henzler, 1993

Streett & Hink (1978) measured an oxygen uptake rate of 8.6 mmol/10E9 cells × d for growing Trichoplusia ni TN–368 insect cells, doubling to 17.3 mmol/10E9 cells × d 14 hr post-infection with wild-type Autographa californica nuclear polyhedrosis virus. Other groups have not determined such a dramatic increase in oxygen consumption rates. Maiorella *et al.* (1988) found similar oxygen uptake rates during exponential growth and 21 hr post-infection at 3.7 mmol/10E9 cells × d and 4.1 mmol/10E9 cell × d, respectively. Sf9 cultures were infected at ca. 2.5 × 10E6 cells ml^{-1} and 20% air saturation with recombinant baculovirus encoding for human macrophage colony stimulating factor (M-CSF). The specific oxygen uptake rate during infection of Sf9 cultures with a recombinant virus encoding for a truncated form of antistasin was determined by Jain *et al.* (1991b) at 3 different DO values (10, 65 and 110%). Experiments were performed in an 8 l stirred tank bioreactor using bubble-free silicone tube gassing to exclude any potential deleterious effects due to sparging. The qO_2 at time of infection, i.e., of uninfected Sf9 cells, and over the whole infection period was significantly increased at the higher DO levels. Values at 65% DO were twice and values at 110% DO four times higher than those measured at 10% DO level. Specific rates remained relatively constant post-infection over 60 hr. Absolute values at 65% DO are comparable to the data reported by Maiorella *et al.* (1988). There was an increase of less than 10% in qO_2 with the maximum rate measured about 20 hr post-infection. Streett & Hink (1978), Schopf *et al.* (1990), Schmid *et al.* (1994), Wong *et al.* (1994) and Hensler & Agathos (1994) likewise observed a maximum qO_2 at 10–20 hr post-infection.

Schmid *et al.* (1994) combined on-line determinations of volumetric uptake rates with off-line hemacytometer determinations of viable cell concentrations to estimate the specific oxygen consumption rates for Sf9 cells during growth and infection with recombinant baculovirus encoding for soluble human IFNγ receptor. After growth at 50% dissolved oxygen with

an estimated qO_2 of 10 mmol/10E9 cells × d, the two identical 25 l airlift bioreactors were maintained at 10 and 50% DO levels during the infection period. In both cases a transient maximum in specific oxygen uptake rates after infection was observed at ≤ 20 hr after the addition of recombinant virus (qO_2 increased by 10%, if conditions remained at 50% DO). This was then followed by a continuous decline in qO_2 values over a 5-day period to a level of about 50% of the pre-infection uptake rates. When the oxygen concentration was lowered to 10% DO, an immediate drop in qO_2 was seen followed by a similar decline phase as recorded for 50% DO during infection. The uptake rates always remained higher for the reactor controlled at the higher DO level. This is in agreement with the data obtained by Jain *et al.* (1991b). As the Sf9 cell diameter increases from 14–16 μm during the exponential growth phase to 19–21 μm at days 2 to 4 post-infection (Schopf *et al.*, 1990; Schmid *et al.*, 1994; Jain *et al.*, 1991b) a continuous decline in qO_2 values was determined when rates were expressed on a per cell volume basis.

Measurements of oxygen uptake rates for Trichoplusia ni (BTI-Tn–5B1–4) cells (Schlaeger *et al.*, unpublished results) during exponential growth at 30% DO and 5.3 × 10E5 cells ml^{-1} gave values of 7.1 mmol/10E9 cells × d. When the viable cell concentrations reached 2 × 10E6 cells ml^{-1}, cultures were infected with recombinant baculovirus (soluble human TNF receptor p55 construct). Post-infection DO levels were controlled at 30%. At 24 and 47 hr after infection qO_2s remained practically unchanged at 7.8 and 7.3 mmol/10E9 cells × d, respectively. Culture characteristics seem to be similar to those of Sf9 cells. By online mass spectrometry Kamen *et al.* (1991) measured a qO_2 of ca. 4.8 mmol/10E9 cells × d during exponential growth of Sf9 cells in a helical ribbon bioreactor (Kamen *et al.*, 1992) using serum-free IPL–41 medium. Later in this batch experiment after cells had been diluted with fresh medium to adjust the cell concentration at about 1 × 10E6 cells ml^{-1} the authors estimated specific consumption rates of 6.5 mmol/10E9 cells × d prior to infection and of 7.7 mmol/10E9 cells ml^{-1} ca. 23 hr post-infection (i.e., a 15% increase).

Other groups have published greater increases in specific oxygen uptake rates. In another study where wild-type virus was used for infection of Sf21 cells, Weiss *et al.* (1982) reported a 25% increase in qO_2 after infection. When Schopf *et al.* (1990) determined off-line consumption rates of T-flask cultured Sf9 cells infected with wild-type and recombinant baculovirus encoding for β-galactosidase at multiplicities of infection (MOI) of 1 and 10 pfu $cell^{-1}$, they observed an increase in qO_2 from 3.4 mmol/10E9 cells × d to a maximum value of ca. 7.4 mmol/10E9 cells × d for recombinant β-galactosidase infected cells. These cells had a nearly 40% higher uptake rate than uninfected cells. For both MOIs the maximum respiratory activity occurred at 16 hr post-infection, followed by a sharp decline. Scott *et al.* (1992) reported increases of 40% in qO_2 from 7 to 9.6 mmol/10E9 cells × d for Sf9 cells infected with a recombinant virus encoding for the core and NS5 regions of hepatitis C virus. Specific oxygen uptake rates (7.9 mmol/10E9 cells × d) for β-galactosidase infected cells (Hensler & Agathos, 1994) increased almost twofold within 24 hr of infection, followed by a gradual decrease until, by 100 hr post-infection, cell respiratory activity had virtually stopped. An average qO_2 of 5.3 mmol/10E9 cells × d was estimated by Wong *et al.* (1994) for uninfected Sf9 cells during exponential growth in a 1 l stirred tank bioreactor using serum-free medium (DO 50%). For infected cultures, the specific oxygen uptake rate increased immediately after addition of virus and a maximum of 1.3 times the value of uninfected cells was noted for all cultures between 8 and 30 hr post-infection, which coincides with the period at which most viral replication and the majority of DNA synthesis takes place. It was observed that the rate of rise in qO_2 decreased as the cell density at the time of infection increased, which meant that the later the infection, the later the maximum qO_2 was observed.

Deutschmann & Jäger (1994) found the qO_2 for Sf21 cells strongly related to cell growth. They calculated a specific oxygen uptake rate of 3.4 mmol/10E9 cells × d for optimal growth at 70% DO. At a DO concentration of 40% and a significantly reduced growth rate (μ = 0.39 day^{-1}), however, the qO_2 value was reduced to 0.7 mmol/10E9 cells × d. Hensler *et al.* (1994) and Hensler & Agathos (1994) like Reuveny *et al.* (1992) reported consistently increased oxygen consumption rates for uninfected Sf9 cells cultivated in serum-free media compared to the same cells grown in serum-containing media. This may have been the result of selecting a cell clone with altered characteristics or reflects a change in metabolic activity of cells in the serum-free environment.

Carbon dioxide production rates and respiration quotients

The respiration quotient (RQ) is a key metabolic parameter, that is independent of cell number or biomass

and can be measured on-line. It reflects the physiological state of cells and is needed to quantify main fluxes of cellular metabolism.

Recently, several publications have appeared in the literature that present data on specific carbon dioxide production rates (qCO_2) and respiration quotients (i.e., the ratio of the specific carbon dioxide production rate divided by the specific oxygen consumption rate) for uninfected and recombinant baculovirus-infected Sf9 cell cultures (Kamen *et al.*, 1991; Archambault *et al.*, 1994; Bedard *et al.*, 1994; Kamen & Tom, 1994). The authors report values of 2.6–7.4 mmol/10E9 cells × d for qCO_2s and values of 1.07 ± 0.06 for RQs in uninfected Sf9 cells cultures. Carbon dioxide production rates increased to 10.3 mmol/10E9 cells × d in virus-infected cultures with respiration quotients determined at 1.12 ± 0.03.

The partial pressure of carbon dioxide in the bioreactor off-gas was monitored by on-line mass spectrometry to estimate specific rates. Some data was obtained under uncontrolled or varying pH and dissolved oxygen conditions (Kamen *et al.*, 1991; Archambault *et al.*, 1994). All of the above studies were performed assuming negligible accumulation of carbon dioxide in the liquid phase, assuming fast carbon dioxide transfer at the gas/liquid interface (i.e., no liquid film resistance) and neglecting the presence of bicarbonate in the culture medium as well as the use of CO_2 for pH control, which will influence the partial pressure of CO_2 in the bioreactor off-gas during the first days of culture. In the light of recent experiments by Bonarius *et al.* (1995), who determined specific carbon dioxide production rates and respiration quotients for the growth of hybridoma cells in bicarbonate-buffered continuous culture (taking into account the above mentioned considerations), a more rigorous investigation into the changes of qCO_2s and RQs of insect cells during growth and infection seems to be indicated. Additionally, disturbances in pH caused by the pulse-wise addition of acid (or CO_2) and alkaline (Royce, 1992) may prove more significant in the case of batch cultures, where the goal would be to exploit the expected small differences in RQ values for control purposes. On-line measurements of respiration quotients of sufficient accuracy may contribute to the process optimization of insect as well as mammalian cell cultures.

Conclusions and outlook

The complete strategy for maximizing the yield of recombinant proteins from insect cell culture must include an optimization of the culture conditions during the growth phase as well as during the subsequent infection phase. The growth of host cells like Spodoptera frugiperda (Sf9 and Sf21) and Trichoplusia ni (BTI-Tn–5B1–4) to cell densities of ca. 10 × 10E6 cells ml^{-1} in batch cultures has so far been achieved. Already today some groups have reported even higher viable cell concentrations (>10 × 10E6 cells ml^{-1}) using nutrient feeding strategies. There will be further improvements in this area. However, probably more important will be the characterization of the optimal physiological state that the cells – at high densities – have to be in at the time of infection so as to maintain the same (or reach even higher) specific productivities than in low-density infections.

The effects of culture conditions (pH, θ, pCO_2, DO, nutrients) during the infection phase on product yield and quality need to be more thoroughly investigated. The cells may potentially have different requirements during the viral replication phase and the following protein production phase. Hardly anything seems to be known about protease activities in Sf9 insect cells (Jäger *et al.*, 1992). Other insect host cells that are currently investigated (Betenbaugh *et al.*, 1991; Davis *et al.*, 1993; Wickham *et al.*, 1992) may have improved characteristics in the respect.

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