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TubeSpin bioreactor 50 for the high-density cultivation of Sf-9 insect cells in suspension

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Abstract Here we present the TubeSpin bioreactor 50 (TubeSpins) as a simple and disposable culture system for Sf-9 insect cells in suspension. Sf-9 cells had substantially better growth in TubeSpins than in spinner flasks. After inoculation with 10^6 cells/ml, maximal cell densities of 16×10^6 and 6×10^6 cells/ml were reached in TubeSpins and spinner flasks, respectively. In addition the cell viability in these batch cultures remained above 90% for 10 days in TubeSpins but only for 4 days in spinner flasks. Inoculation at even higher cell densities reduced the duration of the lag phase. After inoculation at 2.5×10^6 cells/ml, the culture reached the maximum cell density within 3 days instead of 7 days as observed for inoculation with 10^6 cells/ml. Infection of Sf-9 cells in TubeSpins or spinner flasks with a recombinant baculovirus coding for green fluorescent protein (GFP) resulted in similar GFP-specific fluorescence levels. TubeSpins are thus an attractive option for the small-scale cultivation of Sf-9 cells in suspension and for baculovirus-mediated recombinant protein production.

Keywords Baculovirus vector · Bioreactor · Sf-9 cells · Spinner flask · Suspension culture · TubeSpin bioreactor 50

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Introduction

Cultivated insect cells are a commonly used host for the production of recombinant proteins for structure and function studies in conjunction with transgene delivery using recombinant baculovirus vectors (Kost and Condreay 1999; Berger et al. 2004; Kost et al. 2005). Although this approach to protein production is possible with adherent cultures, volumetric scale-up of the culture is more practical with cells in suspension. The suspension cultivation of insect cells is typically performed in spinner flasks, Erlenmeyer-type shake flasks, and various types of bioreactors (Ikonomou et al. 2003). However, spinner and shake flasks are the most commonly used containers for suspension cultures due to economical and practical reasons. Nevertheless, there are disadvantages to their use (Marheineke et al. 1998; Deparis et al. 2003; Batista et al. 2005). Neither is scalable to working volumes greater than about one liter, and spinner

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flasks, in particular, have very poor O_2 transfer into the liquid phase (Nienow 2006). In addition, there is not an adequate scale-down system for either of these vessels, making the performance of medium- and/or high-throughput experiments impractical.

Here we present 50 ml TubeSpin bioreactor 50 (TubeSpins) as an alternative to spinner and shake flasks for the cultivation of insect cells in suspension. These centrifuge tubes fitted with a cap with a membrane filter were originally developed for mammalian cell culture and are suitable for medium- to high-throughput cell cultivation (DeJesus et al. 2004). We describe conditions for the growth of Sf-9 cells in suspension culture in TubeSpins at densities up to 16×10^6 cells/ml. In addition, we tested the capacity of Sf-9 cells grown in TubeSpins to support baculovirus-mediated recombinant protein production.

Materials and methods

Cell culture

Sf-9 cells were adapted to grow in suspension in Sf-900 II SFM (Invitrogen, Basel, Switzerland). For routine maintenance, the cells were cultivated in TubeSpin bioreactor 50 (TPP, Trasadingen, Switzerland) with 10 ml medium and passaged twice per week. The cells were passed by centrifugation at $\sim 1500 \times g$ for 3 min followed by resuspension of the cell pellet in pre-warmed Sf-900 II SFM at 10^6 cells/ml or higher as indicated in the text. The cultures were maintained at 28°C in a shaking incubator (shaking diam. of 2.5 cm and 200 rpm). In 250 ml spinner flasks, the cells were inoculated in 100 ml Sf-900 II SFM at 10^6 cells/ml. The culture was incubated at 28°C at a stirring speed of 75–90 rpm. The cell density and viability were determined by the Trypan Blue exclusion method using a Neubauer hemocytometer. The pH and the concentrations of glucose, lactic acid, glutamine, and ammonium were determined with a Nova analyzer (Nova Biomedical, Waltham, MA).

Baculovirus infection

Sf-9 cells in spinner flasks or TubeSpins were grown to 2×10^6 cells/ml and infected with a recombinant baculovirus coding for GFP (courtesy of Dr. Leona Gilbert, University of Jyväskylä, Finland) (Ojala et al. 2001) at a multiplicity of infection (MOI) of 2–3. Culture samples, 1 ml, were collected at various times post-infection (p.i.), and the cells were lysed by addition of 1 ml ice cold lysis buffer [1.0% (v/v) Triton X-100, PBS] by gentle mixing for 15 min at 4°C (Oker-Blom et al. 1996). The relative GFP-specific fluorescence units (RFU) were determined for cultures of infected and non-infected cells using a fluorescence plate reader with an excitation wavelength of 395 nm and an emission wavelength of 509 nm. The RFU in the medium was measured in a 1:1 mix of Sf-900 II SFM and lysis buffer. The RFU value for each lysate was normalized using Eq. 1:

$$\frac{RFU_{Cells} - RFU_{Medium}}{RFU_{Medium}} \cdot 100\% \quad (1)$$

Results and discussion

Batch cultures of Sf-9 cells in TubeSpins and spinner flasks

TubeSpins and spinner flasks, optimized for Sf-9 cell growth without active aeration, were inoculated in parallel with 10^6 cells/ml. In the former, a maximal of 16×10^6 cells/ml was achieved at day 7 post-inoculation (Fig. 1a), and the cell viability remained above 90% until day 10 (Fig. 1b). In contrast, the cells cultivated in a 250 ml spinner flask only achieved a maximal of 6×10^6 cells/ml (Fig. 1a), and the cell viability dropped below 90% on day 4 and steadily declined thereafter (Fig. 1b). Our results in spinner flasks matched those already reported for Sf-9 and Sf-21 cells (Gotoh et al. 2004; Chiou et al. 2000). By comparison, 8–12 $\times 10^6$ cells/ml have been reported for batch cultures in shake-flasks (Bovo et al. 2008; Batista et al. 2005). Thus the performance of Sf-9 cells in TubeSpins described here was superior to these two widely used suspension cultivation systems. Overall, the highest cell densities for cultivated Sf-9 cells ($\sim 55 \times 10^6$ cells/ml) have only been achieved in fed-batch and perfusion cultures using instrumented bioreactors (Jäger 1996; Bédard et al. 1997; Elias et al. 2000).

Nutrient consumption and waste production for Sf-9 cells in TubeSpins

Glucose is the most important carbon source for insect cell cultivation. In some media, such as

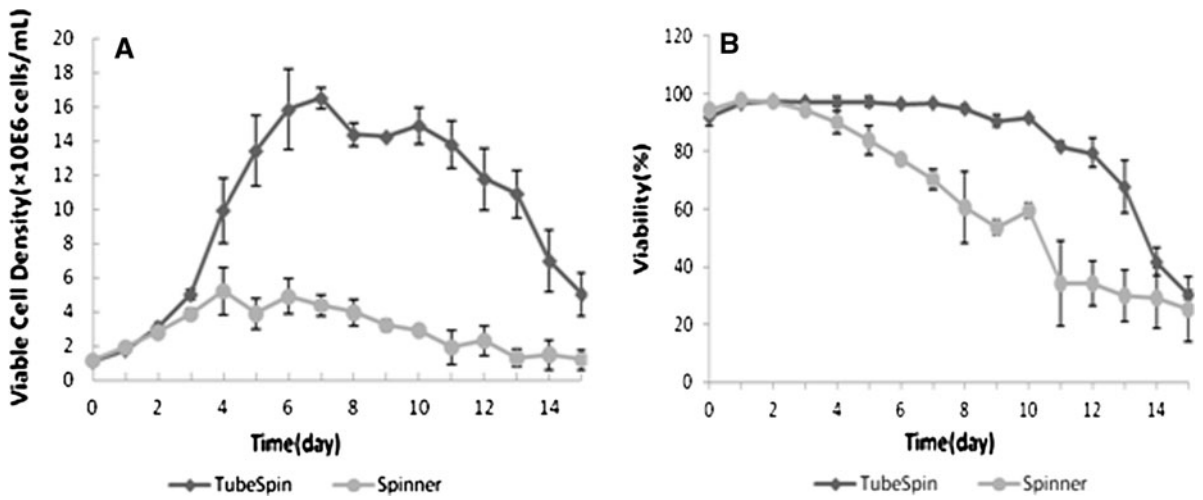

Fig. 1 Growth of Sf-9 cells in batch cultures in TubeSpins and spinner flasks. The cultures were inoculated in duplicate at 10^6 cells/ml. The cell density (**a**) and percentage of viability (**b**) were determined at the times indicated

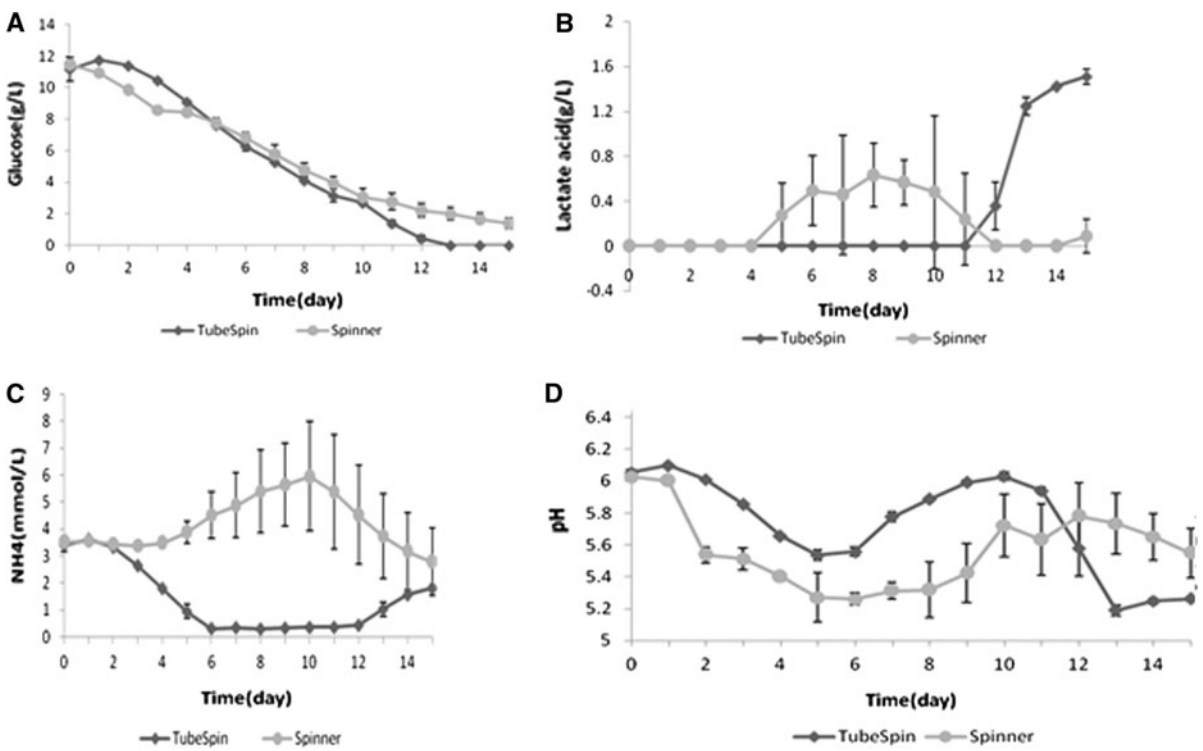

Fig. 2 Comparison of Sf-9 cultures in TubeSpins and spinner flasks. The cultures were inoculated in duplicate at 10^6 cells/ml. Sampling was performed at the times indicated, and glucose (**a**), lactate (**b**), ammonia (**c**), and pH (**d**) were measured with a Nova analyzer

TNM-FM and IPL-41, glucose may be a growth-limiting factor (Bédard et al. 1993; Drews et al. 1995). For the Sf-9 cell cultures in TubeSpins inoculated at 10^6 cells/ml, glucose was depleted from the medium only by day 13 (Fig. 2a), coinciding with the onset of a rapid decline in cell viability (Fig. 1b). For the Sf-9 cells in spinner flasks, the glucose concentration remained above zero throughout the

15-day batch culture (Fig. 2a). Thus, other factors besides glucose contributed to the reduced cell growth and cell viability for the cultures in spinner flasks as compared to those in TubeSpins.

Glutamine consumption was negligible in Sf-9 cultures in both cell cultivation systems (data not shown). Sf-9 and Sf-21 cells can grow in a glutamine-, glutamate- and aspartate-free medium, provided that ammonium ions are present (Öhman et al. 1996). However, glutamine deprivation may result in a reduced cell growth rate.

Cultivated insect cells normally accumulate lactate to very low levels (Bovo et al. 2008). In line with these observations, lactate was only seen after day 12 for Sf-9 cultures in TubeSpins (Fig. 2b). The level of lactate then rapidly increased as the cell viability decreased. For the cultures in spinner flasks, lactate increased from day 4 to a maximum on day 8 and then declined as the cell viability decreased (Fig. 2b). Sf-9 cells do not produce lactate when sufficiently supplied with O_2 even in media with a high initial glucose concentration (Ikonnomou et al. 2003). Thus, the lactate accumulation seen in spinner flasks may have been due to a low level of dissolved O_2 in the culture as predicted in this type of cultivation vessel since it has a low volumetric mass transfer coefficient (Nienow 2006).

Sf-9 cells do not usually produce ammonia during cultivation. Furthermore, the growth of Sf-9 cells is not adversely affected by 10 mM ammonium salts (Bédard et al. 1993). For the cultures in TubeSpins, ammonium declined from initially 3.5 mM to its depletion by day 6 (Fig. 2c). Thereafter, as the cell viability declined, the ammonia level gradually increased (Fig. 2c). The reason for the consumption of ammonia in the early stages of the culture in TubeSpins is not known. For the cultures in spinner flasks, the concentration of ammonia increased to about 6 mM by day 10 before declining (Fig. 2c).

The optimal pH for insect cell culture is between 6.2 and 6.5 because insect tissue fluids normally range from pH 6.2–6.9 (Grace 1962). The pH of the cultures in TubeSpins remained in a pH range between 5.6 and 6.2 until day 12 and then declined to pH 5.2 (Fig. 2d). For the cultures in spinner flasks, the pH was always lower than for those in TubeSpins until day 12, and the minimum pH was 5.3 by day 5 (Fig. 2d). Clearly, the pH was more suitable for Sf-9 cells in TubeSpins than in spinner flasks and may have contributed to the better cell growth performance in the former.

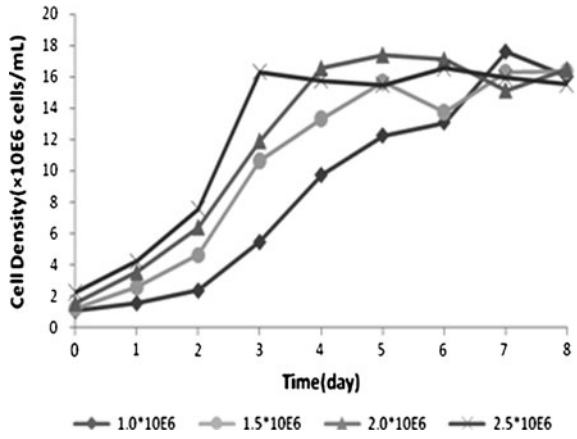

Fig. 3 Effect of inoculation cell density on the growth kinetics of Sf-9 cells in TubeSpin bioreactors. The cells were inoculated at different starting cell densities as indicated. The cell density (**a**) and viability (**b**) were determined at the times indicated by manual counting using the Trypan Blue exclusion method. Each culture was performed in triplicate

Inoculation density for Sf-9 cells in TubeSpins

A long lag phase was observed in the Sf-9 culture in TubeSpins following inoculation at 10^6 cells/ml (Fig. 1a). To determine if this lag phase was dependent on the inoculation density, Sf-9 cells were inoculated with different cell concentrations in TubeSpin bioreactors. The length of the lag phase decreased as the initial cell density increased (Fig. 3). With an inoculation of 2.5×10^6 cells/ml, the culture reached the maximum density of 16×10^6 cells/ml by day 3 while the same cell density was achieved on day 7 for the culture inoculated with 10^6 cells/ml (Fig. 3). The cell viability for the different cultures was similar over the entire cultivation period (data not shown).

Infection of Sf-9 cells with baculovirus in TubeSpins and spinner flasks

To evaluate whether or not the cultivation method affected the efficiency of recombinant protein production, Sf-9 cells in TubeSpins or spinner flasks were infected with a recombinant baculovirus vector coding for GFP (Ojala et al. 2001). Similar levels of GFP-specific fluorescence were observed in the two

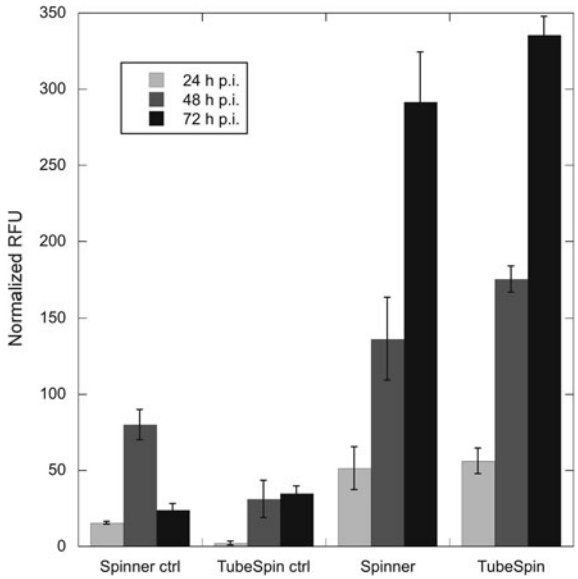

Fig. 4 Comparison of recombinant GFP production in Sf-9 cultures grown in spinner flasks and TubeSpins. The cells at 2×10^6 cells/ml were infected with baculovirus at MOI 2–3 or left uninfected (*ctrl*). The cultures were analyzed for GFP-specific fluorescence at various times post-infection as indicated. For the cultures in spinner flasks, each bar represents the average of two independent experiments, and for the cultures in TubeSpins, each *bar* represents the average of two independent experiments performed in duplicate

cultures at each time point (Fig. 4). Similar results were observed for the infection of Sf-9 cells cultivated in Erlenmeyer flasks (data not shown).

Conclusions

The results show that Sf-9 cells have a better growth performance in TubeSpins than in spinner flasks under the conditions tested here. In batch cultures the maximal cell density was 250% higher in TubeSpins than in spinner flasks, and the cell viability remained above 90% for a longer time in TubeSpins than in spinner flasks. Under similar conditions to those used here, the TubeSpins were shown to have a volumetric mass transfer coefficient (k_La) >17 h^{-1} (Zhang et al. 2009). Such a high k_La assures oxygen supply through passive air transport into the cultivation vessel to support the high cell density observed here. In contrast, spinner flasks have a k_La of about 2 h^{-1} under the conditions used here (Nienow 2006; Diao et al. 2008). The high k_La in TubeSpins also assures that the stripping of CO_2 occurs efficiently. This may be one reason why reason why the pH in TubeSpins over the culture period was more moderate that that observed in spinner flasks. Another major advantage of TubeSpins is that cell cultivation can be performed in volumes as low as 2 ml. Thus a significant savings in medium costs can be realized. Plus, a large number of TubeSpins can be employed at the same time in a small space, allowing many experimental conditions to be tested at once. Moreover, we have shown that Sf-9 cells grown in TubeSpins are suitable for recombinant protein production using baculovirus vectors. Finally, the orbital-shaking technology described here is scalable and has been tested at volumes up to 1,000 l with mammalian cell cultures (Muller et al. 2005; Zhang et al. 2009).

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