Abstract: For large-scale applications in biotechnology, cultivation of mammalian cells in suspension is an essential prerequisite. Typically, suspension cultures are grown in glass spinner flasks filled to less than 50% of the nominal volume. We propose a superior system for suspension cultures of mammalian cells based on orbital shaker technology. We found that "square-shaped" bottles (square bottles) provide an inexpensive but efficient means to grow HEK-293 EBNA and CHO-DG44 cells to high density. Cultures in agitated 1-L square bottles exceeded the performance of cultures in spinner flasks, reaching densities up to $7 \times 10^6$ cells/mL for HEK-293 EBNA cells and $5 \times 10^6$ cells/mL for CHO-DG44 cells in comparison to $(2.5–4) \times 10^6$ cells/mL for cultures of the same cells grown in spinner flasks. For 1-L square bottles, optimal cell growth and viability were observed with a filling volume of 30–40% of the nominal volume and an agitation speed of 130 rpm at a rotational diameter of 2.5 cm. Transient reporter gene expression following gene delivery by calcium phosphate–DNA co-precipitation was the same or slightly better for HEK-293 EBNA cells grown in square bottles as compared to spinner flasks. Reductions in cost, simplified handling, and better performance in cell growth and viability make the agitated square bottle a new and very promising tool for the cultivation of mammalian cells in suspension.

Keywords: serum-free medium; HEK-293 EBNA cells; CHO-DG44 cells; cultivation system; square bottles; orbital shaker

INTRODUCTION

The need for recombinant proteins in fundamental research and for clinical applications is continually increasing. Although other options are available, cultivated mammalian cells are often the primary choice for protein expression when functional protein folding and post-translational modifications are required. For seed trains and for production purposes, mammalian cells are maintained as either adherent or suspension cultures. For the latter, the classical cultivation apparatus at moderate scales is the glass spinner flask with a magnetically driven impeller for mixing. Spinner flasks are less expensive and easier to handle than glass or stainless steel bioreactors. Nevertheless, there is a considerable amount of work associated with their cleaning and sterilization. In addition, the magnetic stirrers and the glass spinners themselves are expensive and require a large amount of incubator space.

The major alternatives to spinner flasks and bioreactors for cultivating mammalian cells in suspension are culture vessels (Erlenmeyer flasks or multiwell plates) agitated on an orbital shaker. Shaken cultivation systems have mainly been developed for bacterial and fungal cultures, but applications for animal cells have also been reported (Girard et al., 2001; Liu and Hong, 2001). For bacterial and fungal cultures grown this way, an important parameter for maximum cell growth is oxygen transfer. In Erlenmeyer flasks under conditions of high cell density, oxygen may become limiting. In spinner flasks, oxygen limitations have been observed in the growth for insect cells (Annathur et al., 2003). Operating parameters that have a considerable impact on oxygen transfer rate and thus on cell growth and viability include the flask size and shape, the orbital shaking speed and diameter, the filling volume, and the surface properties of the flask material (Büchs, 2001; Duetz and Witholt, 2004; Gupta and Rao, 2003; Maier and Büchs, 2001).

Here, we present technology for the maintenance of suspension cultures of mammalian cells in commercially available square-shaped bottles that can be mounted on an orbital shaker within a temperature-controlled chamber. Due to their geometry, square bottles have negative baffles which are expected to increase the surface renewal of the medium, resulting in improved mixing and gas exchange within the cultivation system. This study focuses on 1-L bottles because this corresponds to the size of the most frequently
used spinner flask. We studied the effects of filling volume, agitation speed, and container geometry, size, and composition on cell growth and viability. We found that cultures of HEK-293 EBNAla (HEK-293E) or CHO-DG44 cells maintained in square bottles outperformed cultures grown in spinner flasks. Importantly, the transfectability of HEK-293E cells was not decreased by growth in square bottles.

MATERIALS AND METHODS

Cell Culture

Suspension-adapted HEK-293E cells were grown in serum-free, chemically defined Pro293s-CDM (Cambrex, East Rutherford, NJ) or Ex-Cell 293 medium (JRH Biosciences, Lenexa, KS). Suspension cultures of CHO-DG44 cells were grown in serum-free ProCHO5-CDM medium (Cambrex). Cell lines were routinely maintained in 1-L square bottles made of borosilicate glass (Schott Glass, Mainz, Germany). For comparative purposes, cells were also grown in square bottles composed of polycarbonate (PC), polyethylene terephthalate co-polyester (PETG), or polypropylene (PP) (Nalge Nunc Intl., Rochester, NY). Cultures were incubated at 37°C in a roll-in incubator (Bellco Glass Inc., Vineyard, NY) that was outfitted with a horizontal Model ES-W orbital shaker (Kühner AG, Birsfelden, Switzerland) having a rotational diameter set at 2.5 cm unless otherwise stated. The square bottles and Erlenmeyer flasks with screw caps (Corning, Acton, MA) were fixed onto the shaker with double-sided adhesive transfer tape (3M Corp., Minneapolis, MN). The cells were seeded at a density of 3.5 × 10^5 cells/mL in 300 mL in a 1-L square bottle unless otherwise stated and subcultivated every 3–4 days. The square bottles and Erlenmeyer flasks were tightly closed after inoculation and opened by visual monitoring of the culture. As noted in Materials and Methods, the caps were opened after 24 h of incubation. All experiments to determine the growth characteristics in square bottles of suspension-adapted HEK-293E cells, 1-L square bottles made of glass, PC, PP, or PETG were inoculated at a density of 3.5 × 10^5 cells/mL in Pro293s-CDM medium. For comparison, cells were also cultivated in 1-L Erlenmeyer flasks and in 1-L spinner flasks. The filling volumes were set to 30% of the nominal volume. The agitation speed of the orbital shaker for the square bottles and Erlenmeyer flasks was set to 120 rpm as observations have shown that this condition prevented both the settling of cells and the foaming of the medium. These conditions were also successful in avoiding out-of-phase operating conditions (Buchs et al., 2001). Parallel cultures in the three different vessels were initiated with a single homogeneous cell population maintained in Pro293s-CDM medium in a spinner flask. Cultures grown for 150 h in square bottles achieved higher cell densities ([4.5–5.0] × 10^6 cells/mL) than those grown in Erlenmeyer flasks ([2 × 10^6 cells/mL]) or spinner flasks ([3.5 × 10^6 cells/mL]) (Fig. 1). The material of the square bottle had little effect on cell growth (Fig. 1). Therefore, glass bottles were used in all subsequent experiments because of their lower cost and durability. The glass bottles were also easier to clean and allowed visual monitoring of the culture. As noted in Materials and

Growth Assessment

Cell number was determined using three different methods. Automated counting was performed with either a CASY1 Counter (Scharfe System, Reutlingen, Germany) or a Cedex cell counter (Innovatis AG, Bielefeld, Germany). Manual counting was performed with a Neubauer hemocytometer. Viability was assessed using the Trypan blue dye-exclusion method.

RESULTS

Assessment of Cell Growth in Square Bottles

To determine the growth characteristics in square bottles of suspension-adapted HEK-293E cells, 1-L square bottles made of glass, PC, PP, or PETG were inoculated at a density of 3.5 × 10^5 cells/mL in Pro293s-CDM medium. For comparison, cells were also cultivated in 1-L Erlenmeyer flasks and in 1-L spinner flasks. The filling volumes were set to 30% of the nominal volume. The agitation speed of the orbital shaker for the square bottles and Erlenmeyer flasks was set to 120 rpm as observations have shown that this condition prevented both the settling of cells and the foaming of the medium. These conditions were also successful in avoiding out-of-phase operating conditions (Buchs et al., 2001). Parallel cultures in the three different vessels were initiated with a single homogeneous cell population maintained in Pro293s-CDM medium in a spinner flask. Cultures grown for 150 h in square bottles achieved higher cell densities ([4.5–5.0] × 10^6 cells/mL) than those grown in Erlenmeyer flasks ([2 × 10^6 cells/mL]) or spinner flasks ([3.5 × 10^6 cells/mL]) (Fig. 1). The material of the square bottle had little effect on cell growth (Fig. 1). Therefore, glass bottles were used in all subsequent experiments because of their lower cost and durability. The glass bottles were also easier to clean and allowed visual monitoring of the culture. As noted in Materials and

Plasmids

pEAK8-LH39 and pEAK8-LH41 contain the full-length cDNAs of the light- and heavy-chain genes, respectively, of anti-human Rhesus D IgG under the control of the human EF-1α promoter (Pick et al., 2002). pEGFP-N1 contains the enhanced green fluorescence protein (GFP) gene under the control of the human cytomegalovirus major immediate early promoter (Clontech, Palo Alto, CA).
Methods, the screw cap of the glass bottle was opened one-quarter of a turn after about 24 h of incubation. However, it was observed that if the cap was opened a quarter-turn, a half-turn, or one full turn, the gas exchange was about the same (Gleich and Wurm, unpublished data).

For mammalian cells cultivated in spinner flasks and square bottles, filling volume is critical because the headspace of the vessel functions as a reservoir for oxygen and carbon dioxide. It also affects foam formation caused by the agitation of the medium. In square bottles, if the filling volume is too low, foaming results. To determine the effect of filling volume on cell growth in square bottles, HEK-293E cells were inoculated at a density of 3.5 \times 10^5 cells/mL into square bottles containing 300–700 mL of Pro293s-CDM medium. The square bottles and Erlenmeyer flasks were agitated at 120 rpm, and the spinner flasks were stirred at 80 rpm. Samples were removed from the cultures at the times indicated, and the viable cell number was determined with a CASY1 cell counter.

The growth of the cultures was monitored for three passages. Bottle size did not affect cell growth (Fig. 3). All cultures routinely resulted in cell densities ranging from 4 \times 10^6 to 5 \times 10^6 cells/mL.

The effect of shaking speed on cell growth for a given culture volume was determined by inoculating 1-L square bottles with HEK-293E cells at a density of 3.5 \times 10^5 cells/mL in 300, 400, or 500 mL of Pro293s-CDM. The cultures were agitated at speeds ranging from 100 to 140 rpm, but only the results for the cultures agitated at 120 and 130 rpm are shown (Fig. 4). Overall, better cell growth was observed with agitation at 130 rpm as compared to 120 rpm for all of the volumes tested (Fig. 4). It is known that an increase in agitation speed can improve the rate of oxygen transfer (McDaniel and Bailey, 1969), but in this experiment agitation at speeds higher than 130 rpm resulted in undesired foam formation that was accompanied by a decline in cell viability (data not shown). Agitation at speeds below 120 rpm did not affect the viability of the culture, but the maximal cell density was reduced as compared to agitation at 120 rpm (data not shown). At agitation speeds below 100 rpm, cells settled on the bottom of the bottle.

The experiments described above were carried out on an orbital shaker with a rotational diameter of 2.5 cm. However, most commercially available shakers have an adjustable rotational diameter. To determine the effect of this parameter on cell growth in square bottles, cultures of HEK-293E were agitated on an orbital shaker with a rotational diameter.
diameter of 5 cm. With a filling volume of 300–400 mL, cell growth at an agitation speed of 110 rpm was the same as that at an agitation speed of 130 rpm on a shaker with a rotational diameter of 2.5 cm (data not shown).

Batch Cultivation in Square Bottles

Based on the results from the experiments described above, we compared growth of cultures in square bottles and spinner flasks over an extended time period (8 days). HEK-293E cells were used to inoculate duplicate cultures in 1-L square bottles and spinner flasks at a density of $3.5 \times 10^5$ cells/mL in Ex-Cell 293 medium. Exponential growth was observed for the first 120 h of cultivation in square bottles but only for the first 75 h in spinner flasks (Fig. 5). In square bottles, the maximum cell density reached $(6–7) \times 10^6$ cells/mL whereas the cultures in spinner flasks only achieved a maximum cell density of $4 \times 10^6$ cells/mL by 150 h post-inoculation (Fig. 5). The cell viability of the cultures grown in square bottles remained constant for 150 h post-inoculation at a level greater than 95% (Fig. 5). In contrast, the viability of cultures in spinner flasks began to decrease around 50 h post-inoculation and eventually fell below 85% by 150 h post-inoculation (Fig. 5). Despite the high cell densities, not many cell aggregates were observed in the cultures grown in square bottles. The oxygen level of each culture was analyzed at various times after inoculation. At 72 h post-inoculation, when the cell density of the two cultures was approximately the same, the $pO_2$ levels in square bottles and spinner flasks were 11 and 6 kPa, respectively. Therefore, oxygen limitation did not appear to be a problem for either culture under the conditions tested. The pH of each culture was analyzed through-

**Figure 3.** Effect of square bottle size on cell growth. One-liter, 500-mL, and 250-mL square bottles were inoculated in duplicate with HEK-293E cells at a density of $3.5 \times 10^5$ cells/mL in a final volume of 40% of the nominal volume and agitated at 120 rpm. Each culture was passed at 72 and 168 h after the initial inoculation. Viable cell number was determined using the Trypan blue dye-exclusion method.

**Figure 4.** Comparison of agitation speed on cell growth in square bottles. One-liter bottles were inoculated in duplicate with HEK-293E cells at a density of $3.5 \times 10^5$ cells/mL in different volumes of Pro293s-CDM medium as indicated (300–500 mL) and agitated at 120 or 130 rpm. Viable cell number was determined at 3 days post-inoculation using the Trypan blue dye-exclusion method.

**Figure 5.** Batch cultivation of HEK-293E cells in square bottles. Four 1-L square bottles and two 1-L spinner flasks were inoculated with HEK-293E cells at a density of $3.5 \times 10^5$ cells/mL in 300 mL of Ex-Cell 293 medium. The square bottles were agitated at 130 rpm, and the spinner flasks were stirred at 80 rpm. The cell number for the cultures in the square bottles labeled “1” was determined with a Cedex cell counter. For the cultures in the square bottles labeled “2” and in the spinner flasks, the viable cell number was determined manually with a Neubauer hemocytometer. The average viability of the cultures in square bottles (1 and 2) and of the spinner flasks was determined using the Trypan blue dye-exclusion method.
out the cultivation period. The pH at the time of inoculation was 7.2. By 72 h post-inoculation, the pH decreased to approximately 6.8 in spinner flasks and 6.5–6.7 in square bottles.

**Transfection of Cells Grown in Square Bottles**

Our major objective for the cultivation of mammalian cells in square bottles was to provide large numbers of cells with a high viability for large-scale transient gene expression. It was important to determine if cells grown in square bottles could be transfected with the same efficiency as those grown in spinner flasks because it has been shown that some operational changes or media additives appear to alter specific productivity by impacting gene transcription, cell cycle progression, or cellular metabolic activity (Brorson et al., 2002). HEK-293E cells grown in either square bottles or spinner flasks were used to seed 12-well microtiter plates. The cells were then transfected with pEAK8-LH39 and pEAK8-LH41, encoding the light- and heavy-chain IgG genes, respectively. The transfection mix also included pEGFP-N1 (2% of the total DNA) to provide a visual control of the transfection efficiency. At 3 days post-transfection, the level of IgG expression from cells grown in square bottles was slightly higher than that observed in spinner flasks (Table I). GFP expression was approximately the same for the two cultures (Table I). These results demonstrated that HEK-293E cells grown in a shaken square bottle cultivation system were efficiently transfected.

**Growth of CHO-DG44 Cells in Square Bottles**

To determine if cell lines other than HEK-293E could be grown in square bottles, 1-L bottles and spinner flasks were inoculated in duplicate with CHO-DG44 cells at a density of $3.5 \times 10^5$ cells/mL in ProCHO5-CDM medium at a filling volume of 300–400 mL. As observed with HEK-293E cells, growth of CHO-DG44 cells in square bottles over several passages was superior to that observed in spinner flasks (Fig. 6). The cultures in the square bottles routinely reached a cell density of about $5 \times 10^6$ cells/mL, while the cell density in spinner flasks was 2.5 to $4 \times 10^6$ cells/mL (Fig. 6). As for HEK-293E cells, a study of cell growth over an extended time period (7 days) was performed with CHO-DG44 cells. Duplicate cultures in 1-L square bottles and spinner flasks were inoculated at $2.5 \times 10^5$ cells/mL. Cultures in square bottles reached a higher cell density than those in spinner flasks (Fig. 7). The maximum cell density for the cultures in spinner flasks was about $2.5 \times 10^6$ cells/mL, whereas the cultures in the square bottles reached nearly $5 \times 10^6$ cells/mL (Fig. 7). Importantly, for the cultures in square bottles, the viability remained above 95% for a 4-day period and above 90% for a 1-week cultivation (data not shown). The results demonstrated that the culture system described here is applicable to cell lines other than HEK-293E.

**DISCUSSION**

Square bottles mounted on a horizontal orbital shaker were evaluated with respect to their potential for the propagation of suspension cultures of HEK-293E and CHO-DG44 cells. For these two widely used cell lines, very good cell growth, which depended on specific conditions for the filling volume and the agitation speed, was observed. In fact, maximal cell densities in regular subcultivations exceeded the densities observed with cultures grown in spinner flasks, the standard system to cultivate cells using a non-instrumental approach. We also found that there is a high level of reproducibility in performance as the cell mass and viability of parallel cultures usually differed by less than 10%.

Filling volume and shaking speed, each highly dependent on the other, were found to be among the most important

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**Table I.** Reporter protein expression in transfected HEK-293E cells.

<table>
<thead>
<tr>
<th>Cultivation system</th>
<th>IgG expression a (mg/L)</th>
<th>GFP expression b (RFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinner flasks</td>
<td>$3.5 \pm 0.6$</td>
<td>$4900 \pm 300$</td>
</tr>
<tr>
<td>Square bottles</td>
<td>$4.0 \pm 0.7$</td>
<td>$4500 \pm 200$</td>
</tr>
</tbody>
</table>

a Determined by ELISA at 3 days post-transfection. Each value is the average of 3 transfections.
b Determined by fluorometry at 3 days post-transfection. Each value is the average of 3 transfections.
parameters for optimal cell growth in square bottles. For a 1-L bottle, a filling volume of 400 mL and a shaking speed of 130 rpm were shown to be optimal when using a rotational diameter of 2.5 cm. However, other combinations of filling volume and shaking speed also resulted in good cell growth. For applications requiring expansion to a large-scale culture, a filling volume of 500 mL in a 1-L bottle is possible, but the maximal cell density may not be as high as observed for the optimal conditions described here. It is also possible to increase the volume of the culture by using larger bottles. Besides the experiments described here with bottles having volumes between 250 mL and 1 L, we have also been successful in cultivating cells in 2-L and 10-L square bottles. In addition, growth to a high cell density with high viability can be achieved in small volumes (0.125–2.0 mL) in microtiter plates (Duetz and Witholt, 2001; Girard et al., 2001; Strobel et al., 2001) and in larger volumes (up to 56 L) in various containers (Liu and Hong, 2001) when the mixing parameters are optimally adjusted.

The agitation of suspension cultures by orbital shaking can improve gas exchange into and out of the medium (Büchs, 2001; Girard et al., 2001), and the geometry of square bottles may further enhance gas exchange (Hermann et al., 2002). The corners in square bottles may act similarly to baffles in bioreactors, increasing shear stress, speed distribution, and surface renewal, resulting in a more turbulent flow and in a significant increase in the oxygen transfer rate (OTR). An optimal surface-to-volume ratio in square-shaped vessels may also lead to turbulent flow (Duetz and Witholt, 2001). For shaken vessels, the ratio of the volume of the liquid to the volume of the headspace in combination with the speed of agitation may limit the OTR or result in out-of-phase mixing conditions, which in turn influence the efficiency of agitation (Büchs et al., 2001; Duetz and Witholt, 2001). This may explain why drastically reduced cell growth was observed for culture volumes between 600 and 800 mL in a 1-L square bottle despite the partial opening of the bottle (one-quarter turn of the cap) and the absence of out-of-phase operating conditions. Although it has been shown in the past that the type of closure can inhibit or reduce growth rates in Escherichia coli (Gupta and Rao, 2003; McDaniel and Bailey, 1969), this was not the case when different caps and size openings were tested under our optimal conditions.

Figure 7. Batch cultivation of CHO-DG44 cells in square bottles. One-liter square bottles and spinner flasks were inoculated in duplicate at $2.5 \times 10^5$ cells/mL in 300–400 mL of ProCHO5-CDM medium. The square bottles were agitated at 130 rpm, and the spinner flasks were stirred at 80 rpm. The viable cell number was determined at the times indicated using the Trypan blue dye-exclusion method.
There are many benefits to using glass rather than plastic bottles. They are economical, easy to clean, and allow for visibility of the culture. Additionally, these bottles were the easiest to fit onto the orbital shaker with double-sided tape. There are other means of fixing the vessels, such as within racks attached to the shaker. This approach was evaluated, but the double-sided tape was easier to handle and allowed for the optimization of incubator space. As for bottle reuse, the PC and PP bottles did not always survive a second or third round of autoclaving and were therefore not as durable as the glass bottles. The single-use PETG bottles were not cost efficient.

The main purpose of this research was to optimize a cultivation system for producing large quantities of mammalian cells in suspension for large-scale transient gene expression. We have shown that cells cultivated in square bottles were transfected with the same efficiency as cells grown in spinner flasks. Cultures in square bottles not only yielded a higher number of cells per unit volume than cultures in spinner flasks, but the quality of the cells as measured by viability was also better. Cultures grown in square bottles had viabilities higher than 90% for up to a week after inoculation. We have found that this parameter of culture quality can be predictive of success for large-scale transient gene expression in suspension cultures. Although we currently perform large-scale transfections in spinner flasks or bioreactors (Girard et al., 2001; Meissner et al., 2001), we are presently investigating methods for direct transfection of suspension cultures in square bottles.

CONCLUSIONS

Superior growth of suspension-adapted HEK-293E and CHO-DG44 cells was observed in agitated square bottles as compared to spinner flasks. Cell viability was maintained above 90% for as long as 7 days after inoculation into square bottles, making cell passage once per week a possibility. Following transfection by calcium phosphate-DNA co-precipitation, cells grown in square bottles yielded the same or slightly higher levels of recombinant protein than did cells grown in spinner flasks. Additional advantages of this cultivation system include its low cost and the ease of cleaning and handling the bottles. Thus, agitated square bottles represent an attractive alternative to other systems for the cultivation of mammalian cells in suspension.

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References


