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Tools for High-Throughput Medium and Process Optimization

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Summary

There is an increasing need for high-throughput scale-down models that are representative of commercial bioprocesses, particularly in the field of animal cell biotechnology. In this chapter we describe two protocols for small-scale disposable plastic vessels: 125-mL Erlenmeyer shake flasks and 50-mL centrifuge tubes. We also describe two common applications for these scale-down platforms: (1) satellite cultures derived from conventional bioreactor runs for optimizing the feed strategy for fed-batch cultures and (2) testing multifactorial experimental designs for designing the components of a base medium.

Key Words: Cell culture; Chinese hamster ovary; high-throughput; fed-batch; media; factorial.

1. Introduction

The use of animal cells as the leading host system for the production of recombinant proteins requires the use of high-throughput technologies in order to quickly establish production processes. Process development timelines can only be met with intensive screening for high-producing clones followed by medium screening and process optimization experiments, typically carried out in small-scale culture systems. The classical spinner flask culture vessel is still widely used today *(1)*. However, spinner flasks and small-scale bioreactors need relatively large culture volumes (0.5–3 L) and are not ideal for a truly high-throughput platform. With the availability of (1) new shaking incubators, providing humidity and a CO_2-controlled environment, and of (2) sterile disposable culture vessels (**Figs. 1–3**), shake technology (used for decades in microbiology research) has rapidly become a standard approach in process development for mammalian cells *(2,3)*.

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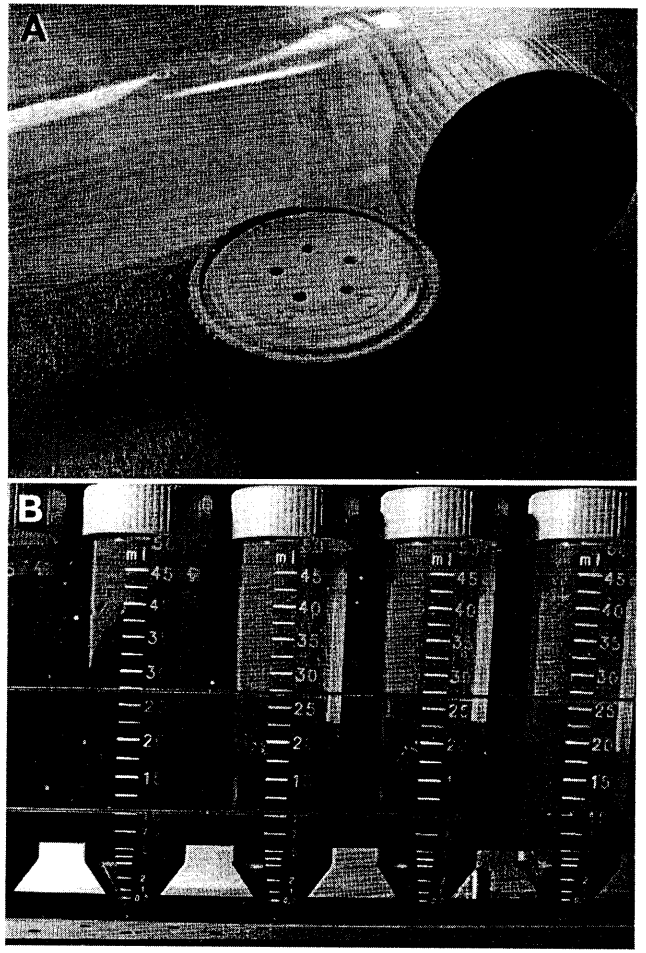

Fig. 1. Disposable 50-mL culture tubes. (**A**) Optimized filter caps with defined permeability for gases and humidity (similar caps are available for shake flasks). (**B**) Tubes with different volumes of culture, shaken at 200 rpm.

Two disposable culture vessels will be described in this chapter: polycarbonate Erlenmeyer flasks and polypropylene tubes. While the 125-mL Erlenmeyer flasks are well established in many companies, the 50-mL tubes are not well known by most users. Both culture vessels have many features in common and show similar performance characteristics. However, the 50-mL tube is the most flexible culture vessel available today **(Fig. 2)** because the working volume can be varied within the range 1–40 mL. A working volume of 5 mL is most convenient since it facilitates many experiments with only 100 mL of cell culture inoculation, and there is still enough culture volume available for sampling and

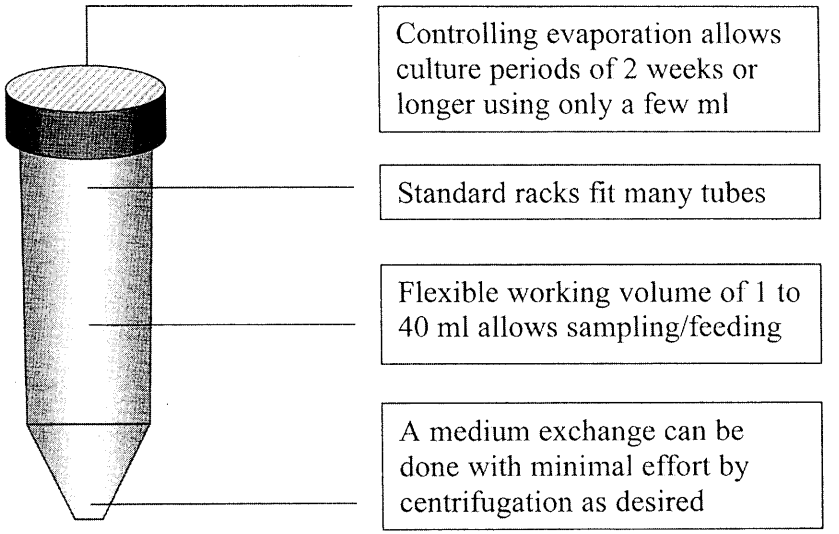

Fig. 2. Advantages of the 50-mL disposable tube as a cell culture system.

analysis *(4)*. Typical shake flask sizes are 125 mL (with 30- to 40-mL working volume) or 250 mL (with 70- to100-mL working volume); both are available presterilized with vented caps for gas exchange.

2. Materials

2.1. Shake Flask Experiments

1. A shaking incubator with CO_2, temperature, humidity control, and with clamps for 125-mL shake flasks, e.g., from Kuhner A.G., Switzerland **(Fig. 3)**. Set the orbit radius (throw) at 2.5 cm (*see* **Note 1**), rotation at 130 rpm, temperature at 37°C, atmosphere of 5% CO_2 in air, and the relative humidity at 80% (*see* **Note 1**).
2. A centrifuge with a rotor arm and buckets for 50-mL centrifuge tubes.
3. Corning sterile polycarbonate shake flasks (125 mL) with vented caps to allow gas exchange and prevent contamination.
4. Basal medium (e.g., Dulbecco's modified Eagle's medium [DMEM]/F12).
5. A cell-counting device (e.g., Neubauer hemacytomer and microscope or an automated device such as the Beckman Vi-Cell).
6. For large experiments, software for experimental design and analysis, e.g., Design Expert from Stat-Ease *(5)*.

2.2. Shaken Tubes

1. A shaking incubator with CO_2, temperature, and humidity control with holders for 50-mL centrifuge tubes (e.g., from Kuhner A.G., Switzerland). Set the orbit radius

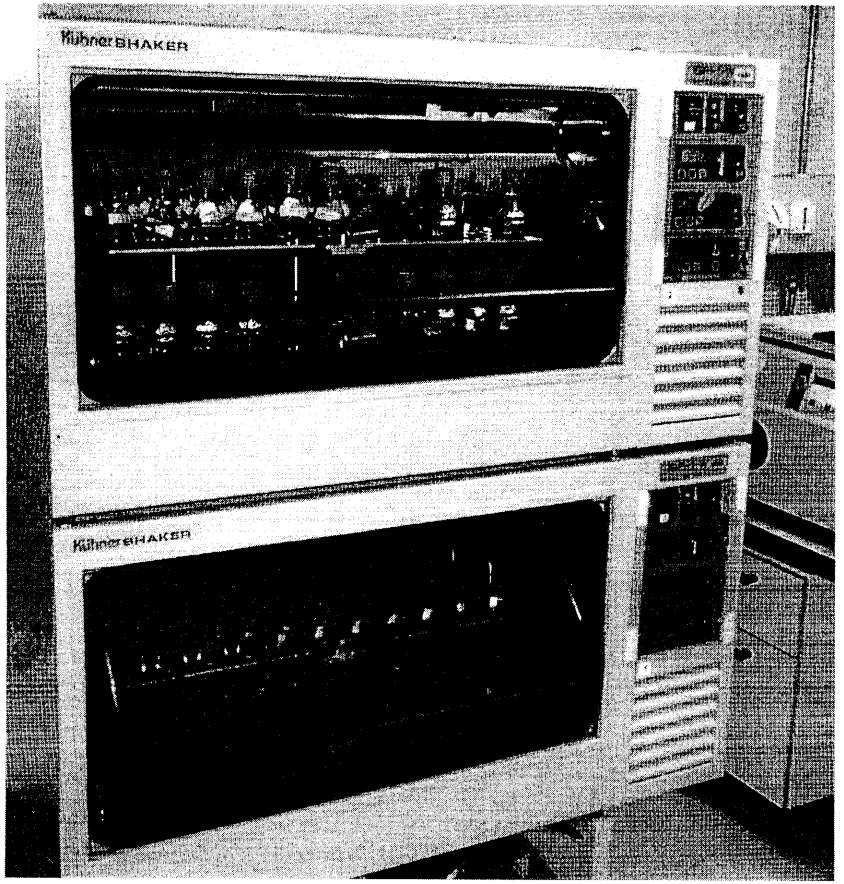

Fig. 3. 125-mL shake flasks in the Kuhner shaking incubator with temperature, humidity, and CO_2 control.

(throw) at 5 cm (**Note 1**), rotation at 200 rpm, temperature at 37°C, atmosphere of 5% CO_2 in air, and relative humidity at 80%.
2. A centrifuge with a rotor arm and buckets for 50-mL centrifuge tubes.
3. Techno Plastic Products (TPP) 50-mL sterile polypropylene tubes with vented filter caps (**Fig. 1**).
4. A cell-counting device (e.g., Neubauer hemacytomer and microscope or an automated device such as the Beckman Vi-Cell).

3. Methods

3.1. Multifactorial Experiments in Shake Flasks

Choosing the right factors to be tested in each experiment is recognized as the most difficult part of the experiment. The actual number of factors tested depends on the objectives of the experiment and the knowledge that is already available for a given cell line. A large number of factors (>10) is typically used to screen different media or clones, whereas for optimization purposes (e.g., dose-response curves and optimization of factors) typically <10 factors are evaluated. A factor can be one single chemical compound (e.g., glucose, glutamine) or a group of compounds (e.g., essential amino acids, vitamins) or physical parameters (e.g., pH, temperature). Many different factors have been described in the literature that have positive effects on productivity and/or cell growth ***(6)***, and a comprehensive literature search is recommended before embarking on this type of study.

1. Design the experimental treatments and flask plan. Although this can be done manually, it is easier to use specialist software for experimental design such as Design Expert from Stat-Ease (*see* **Note 2** and **ref. 5**). The experimental plan in **Table 1** shows an example of a factorial experiment testing five factors and their interactions, which uses 16 shake flasks.
2. Prepare concentrated solutions of each factor according to the flask plan and add the base medium to achieve a constant volume of 31.5 mL per shake flask. Incubate at room temperature overnight to check for sterility.
3. Take cells from the seed-train culture for inoculation at working day 0 (*see* **Note 3**). Centrifuge cells at 200*g* for 10 min at room temperature and resuspend cells in the base medium to make a 10-fold cell concentrate; e.g., for a cell seeding density of 0.2 million viable cells per mL make a concentrated solution of 2 million viable cells per mL. Inoculate each flask with 3.5 mL of this 10-fold cell concentrate to achieve a final volume of 35 mL per shake flask.
4. Incubate for the duration of the experiment in the shaking incubator (set at 130 rpm, 80% humidity, 5% CO_2, and 37°C). For experiments where only cell growth is measured 7 d is usually sufficient, but a longer period (e.g., 10–12 d) may be required to measure protein production or cell viability at the end of a culture.
5. Samples are taken under sterile conditions for cell counts and other measurements every 2–3 d for the duration of the experiment. For each sample (typically 1–2 mL of culture) perform cell counts, either manually using trypan blue staining or using an automated device such as the Beckman Vi-Cell.
6. If protein productivity data are important throughout the culture, cells are separated from the culture supernatant by centrifugation (200*g*, 10 min at room temperature) and either enzyme-linked immunosorbent assay or an alternative protein quantitation assay (e.g., Biacore) is performed on the supernatants. Metabolite data may also be measured at this stage, if required (*see* **Note 4**). We have found that up to 10 mL of samples can be taken from each flask without adversely affecting the culture.
7. Alternatively, in simple experiments protein and metabolite data are only measured on the final day of the experiment.
8. At the end of the experiment the quantitative data (e.g., cell counts, protein levels, and metabolite levels) are fed back into the Design Expert or equivalent software. Note that that each response requires a separate data column and is analyzed independently. An example of one such response is shown in **Fig. 4**, and a normal

Table 1
Experimental Plan and Viable Cell Results for a Multifactorial Experiment in Shake Flasks, Testing the Interactions Between Five Media Components (Factors A-E)

Flask	Factor A	Factor B	Factor C	Factor D	Factor E	Viable cells (million per mL)
1	1	1	1	1	1	3.15
2	0	1	0	0	0	2.04
3	0	1	1	0	1	3.41
4	0	0	0	1	0	3.14
5	1	1	0	1	0	1.30
6	0	0	1	0	0	2.31
7	1	0	0	1	1	3.63
8	1	0	0	0	0	1.57
9	1	1	1	0	0	1.09
10	0	0	0	0	1	3.92
11	1	1	0	0	1	2.32
12	0	1	0	1	1	4.08
13	0	0	1	1	1	3.97
14	0	1	1	1	0	2.40
15	1	0	1	1	0	2.09
16	1	0	1	0	1	2.50

0 = factor is absent; 1 = factor is present. Viable cell counts were obtained using the Beckman Vi-Cell on working day 9 of the experiment.

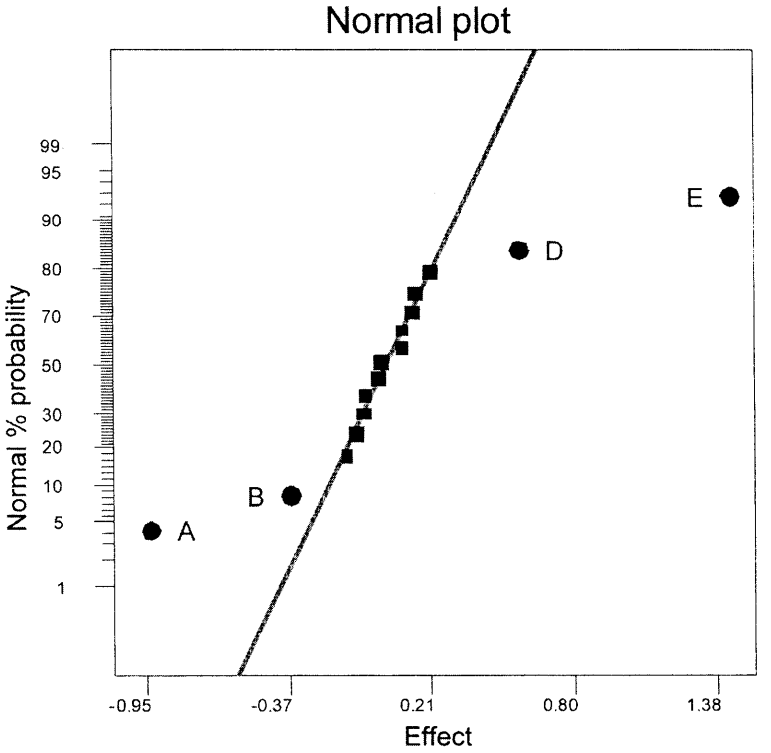

Fig. 4. Normal plot of the results shown in **Table 1**. Significant factors are shown in circles (**A** and **B** are negative, **D** and **E** are positive). Nonsignificant factors (**C**) and their interactions are shown in squares.

plot of the five factors analyzed in a multifactorial experiment on CHO cells using the flask plan is shown in **Table 1** (*see* **Note 5**).

9. It is good practice to perform a repeat part of experiment in triplicate shake flasks to ensure reproducibility. Typically, we repeat the best two to three flasks from the original experiment in triplicate and also include triplicate flasks of the best combination predicted by the Design Expert model. A negative control (i.e., base medium only) is also included in triplicate flasks.

3.2. Optimization of a Fed-Batch Process in Shaken Tubes

A useful application of the 50-mL shaken tube platform is the optimization of a fed-batch process (*see* **Fig. 5**). The batch process is started in a single bioreactor, from which the cells are taken and distributed into 50-mL tubes where candidate feeds are tested in parallel. Feeds are usually added toward the end of the exponential growth phase. However, it is recommended to test different time points in order to define the optimal feeding strategy.

1. Define the 20 feeds and prepare the different feed stock solutions. Fed volumes should be no more than 10% of the total volume (e.g., 0.5 mL in a final volume of 5.5 mL). Include a negative control, to which no feed is added.
2. The feed stock solutions are loaded into sterile 50-mL centrifuge tubes that are clearly labeled. Three series of 20 tubes are prepared in order to test the effects of adding the feeds at three different time points (*see* **Note 6**).
3. The first set of feeds is tested after a few days in the bioreactor with cells that are still growing. One hundred milliliters of cell culture is taken from the bioreactor and distributed in 5-mL aliquots into the 20 tubes (*see* **Note 7**).
4. Tubes are shaken at 200 rpm in the Kuhner incubator (80% humidity, 5% CO_2, 37°C) until the end of the experiment (*see* **Note 8**).
5. A second set of 20 tubes is launched when the cells are at the transition between the exponential growth and stationary phase of culture, and the third set of tubes is launched 24 h later.

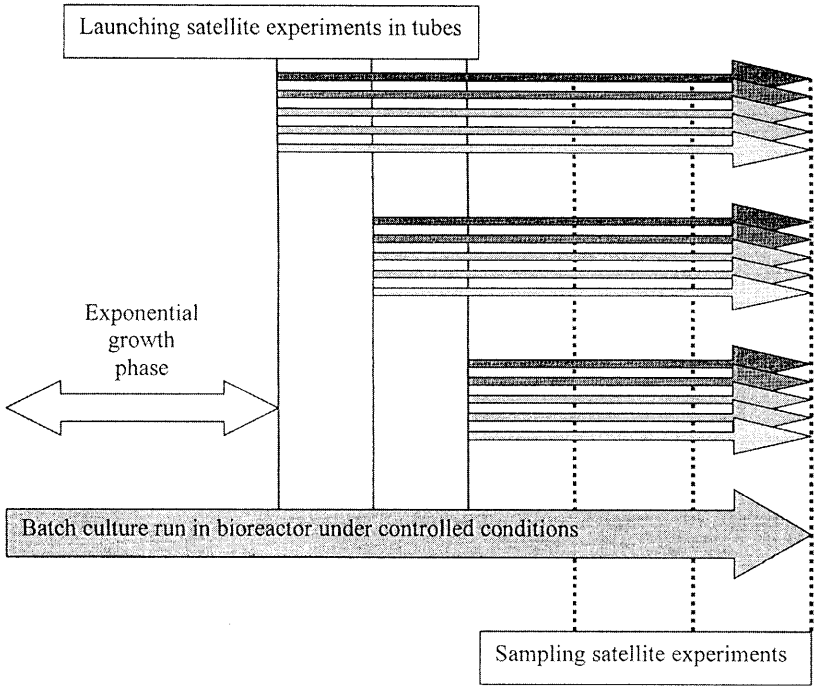

Fig. 5. Process optimization by satellite experimentation in shaken tubes, as described in **Subheading 3.2.**

6. Two to three days before the end of the experiment a sample is taken and analyzed for viable cell number, product concentration, and other parameters of interest, such as metabolites (*see* **Note 4**). The sample volume can be up to 2 mL without inducing any adverse effects in the remaining culture.
7. At the end of the experiment the whole tube is harvested and used for cell counts and other types of analysis (*see* **Note 9**).

3.3. Conclusions

In addition to the shake flask and shaken tube platforms described here for high-throughput screening, other types of vessels can be used. At the micro scale, animal cells have been successfully grown in conventional plastic plates, e.g., in 96- or 24-well plates *(7)*. Alternatively, cells can be grown to high densities in shaken deep-well microtiter plates, although precautions need to be taken to avoid excessive evaporation *(8)*. Working at small volumes (less than 1 mL per well) also limits the amount of sample that can be taken for analysis, so identical plates are often set up in parallel in order to overcome this constraint. Process development can also be performed in small-scale bioreactors of working volume 1-5 L *(9)*, although such a platform cannot be run in a high-throughput mode.

4. Notes

1. Various orbit radii (throws) were tested using the 50-mL tubes, and we observed that a throw of 5 cm was optimal for keeping the cells perfectly in suspension. Similarly, a throw of 2.5 cm proved optimal for cells in 125-mL shake flasks.
2. The statistical techniques of multifactorial analysis and response surface design are explained elsewhere *(10,11)*. There are a number of software packages available to assist the researcher in experimental design and analysis, such as Design Expert from Stat-Ease *(5)* or MODDE from Umetrics *(12)*. We highly recommend these techniques for large experiments (when more than three variables are tested simultaneously).
3. If the medium used for growing the cells for inoculation is a richer formulation than the ones being evaluated, one should be aware of potential carryover effects, where cells are still responding to factors in the previous medium. Such carryover effects may mask a lack of essential components in the test medium for two to three cell passages (about 5–7 d). Therefore, for testing lean media we recommend growing the cells in the test medium for 5–7 d prior to the start of the experiment.
4. In addition to cell counts and protein productivity measurements, useful information can be gained about the cell's metabolism by testing the supernatants on a metabolite analyzer, e.g., a Nova Bioanalyzer, which measures glucose, lactate, glutamine, and ammonia.
5. In this analysis (**Fig. 4**) factors that do not lie on the median line (circles) have a significant effect on the viable cell concentrations measured on day 9 of the experiment. Factors to the right of this line have positive effects, and factors to the left of the line have negative effects. Note that not all the responses will be the same, e.g., factors that are positive for cell growth may not be positive for protein production.
6. Depending on the stability of the feed solutions, this can be done at the beginning of the experiment. The solutions are then stored at 4°C until they are needed. Alternatively, unstable feed solutions are distributed into the tubes immediately before the cells are added.
7. Since the tubes already contain the feed solutions, the distribution of the cells should be fast. It is important that this does not take too much time, otherwise the cells will start settling, and it is critical that all the tubes get the same number of cells.
8. Five percent CO_2 in air will guarantee a physiological pH if the bicarbonate concentration of the medium is within the range 12–18 m*M*. In small-scale bioreactors (with a few liters working volume), bicarbonate can be completely utilized. This occurs when CO_2 is stripped out of the medium via aeration and the pH controller adds HCl or cells produce equivalent amounts of lactate. In bicarbonate-deficient media the pH drops below 7 in the presence of 5% CO_2. In such cases (if the bicarbonate can be measured) it can be easily added back or the CO_2 level of the vessel can be lowered. Alternatively, 20–30 m*M* hydroxyethyl piperazine ethane sulfonate

(HEPES) can be included from the beginning of the experiment to maintain pH. Although the pH might still change in the presence of HEPES, the medium is much less sensitive to changes in bicarbonate or CO_2.

9. In general, both shake flask and shaken tube data correlate well with bioreactor data. Nevertheless, these platforms should be regarded as high-throughput screening tools that facilitate the testing of many different media and feed components in parallel. For industrial purposes, all results should be confirmed in a bioreactor that is representative of the final process.

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Cell Characterization and Analysis