Commentary

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# Scale-up and predictability in process development with suspension cultures of mammalian cells for recombinant protein manufacture: comments on a trend reversal

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In the past (1980–2000), 'non-instrumented' small-scale systems for the suspension culture of mammalian cells disappointed when used for the development of processes that needed to be applied in controlled stirred tanks. That is, cell growth and productivity of cells was usually better in the larger pH/oxygen-controlled reactors. In more recent years, data from cultures as small as 10 ml, in non-instrumented systems, are frequently better (higher yield, viability and cell density) than the ones seen from cultures in well-controlled bioreactors (volumetric yields for recombinant proteins from CHO cells in bioreactors in general have improved by 20–40-fold over the period of 25 years). Why is this? The commentary here tries to explain, at least partially, the observed trend reversal.

Large-scale suspension cultures for the manufacture of recombinant protein therapeutics have been used since the early 1980s, with human recombinant tissue plasminogen activator being the first protein made at the 10,000 l scale by stably transfected and gene-amplified CHO [1].

A most important step towards large-scale use of CHO cells was the generation of suspension-adapted subpopulations, derived from adherent cultures with serum. Development of media and generation of populations of cells in serum-free culture was executed in 'spinner flasks', unique glass bottles ranging in volume from 100 ml to up to 5 l [2–4]. The 500-ml spinner FLASK was the most popular, used with cell cultures of up to 200 ml. Spinner flasks exhibited a flat Teflon® impellor with a magnet at its lower end. A 'spinner-base' transferred a magnetic force through the glass body of the spinner to the impellor [101]. Spinning rates of 30–50 rpm assured a gentle movement of the suspension culture. The bottles did not have baffles, for fear that too high a shear stress would be exerted. The headspace over the liquid was considered sufficient to supply oxygen to the cells. During the later phases of the culture, when higher densities were expected, the two access ports to the flask could be 'cracked open'. Thus, diffusive entry and exit of gasses was provided along the space of the winding of the cap. In a warm room (37°C, no $CO_2$), available to one of the authors (Wurm), up to 40 flasks could be used for the screening and identification of favorable process conditions.

Two aspects of these spinner bottles have held back efficient optimization of processes. First, running more than 30 spinners by a single operator was impossible, both in terms of generation of seed cultures (large volumes) and in terms of workload. Second, severe oxygen limitations for cell densities higher than $3 \times 10^6$ cells/ml was 'overlooked' or neglected (including by Wurm) for a long time.

Attempts for 'high-throughput' cultures with disposable multiwell plates (6-/12-/24-well; static or slowly shaken) were unsatisfactory, mostly because of 'edge

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effects' and water evaporation, the latter having profound effects on the osmolarity of the culture. Also, these 0.5–3 ml cultures were not providing enough sampling volumes for production phases that could last 7 days or longer.

Frustration in cell culture development laboratories was widespread in the 1980s and 1990s. Companies invested heavily into fully instrumented, stirred bioreactors, the smallest of which had a minimum working volume of approximately 0.7 l with a price tag of up to US$100,000/piece. Laboratories were installed with 30–100 (or more) of these, with the corresponding cost in investment, human resources and maintenance.

Recognizing the serious limitations in small-scale systems, three trends for cell culture development efforts emerged. The first was to 'multiplex' bioreactors: 0.2–0.5 l working volume, controllable bioreactors were linked together and made moderately more affordable and easier to use for a non-expert user. Recently, individual vessels of multiplexed systems are provided as single-use containers, avoiding labor-intensive cleaning and sterilization.

Since the multiplexed stirred bioreactors could not deliver high-throughput capability, a second development was initiated with the 'SimCellMicroBioreactor Array Technology' [5]. This is a robotized µl-scale bioreactor system with control of pH and oxygen. It runs hundreds of mini bioreactors simultaneously. Mixing is mediated by a small gas bubble within the reactor volume (0.7 ml), while the entire array (each containing six chambers) is turned vertically on a wheel. However, the SimCell system came with a rather prohibitive cost of approximately $1 million and has therefore found only limited entry into process development activities with mammalian cells.

The third approach was to adopt shaking (from microbiology) as the mixing principle, and to reduce the working volume to the milliliter scale.

While these three approaches were pursued, an insight was spreading on 'industrialized' cell lines, such as CHO, NS0 and HEK-293 cells in their optimized media, that they are not that shear sensitive. More robust approaches for scale-down systems of cell culture process development were tried. De Jesus had successfully applied, since the late 1990s, 50-ml centrifuge tubes as cell culture vessels and had used 20–100 of these simultaneously for screening purposes. Eventually, out of this work, together with a commercial plastic ware provider, the 'TubeSpin bioreactor' was developed [6]. Being slim cylinders, these non-instrumented 'bioreactors' have a conical bottom, a cap with ventilation holes (secured with a gas-permeable membrane) and can be used with working volumes of 5–30 ml. They are to be mounted vertically onto a shaker platform and shaken orbitally at 150–300 rpm (displacement radius 25 mm). In a single incubator shaker more than 400 tubes can be run and two to three operators can execute work with 1000 bioreactors [7].

In spite of high liquid velocities in these reactors, no signs of shear stress on cells could be observed. The gas-transfer rates in these reactors are, under cell culture working conditions, 5–10-times higher than those seen in any small- (or large)-scale stirred tank bioreactor for mammalian cells [8]. This explains why these small reactors supply sufficient oxygen, via passive transport across the membrane in the cap, even for high cell densities ($2 \times 10^7$ cells/ml).

Another system utilizing orbital shaking, and 'borrowed' from microbial fermentations, is the 'classical' Erlenmeyer bottle. For cell culture applications, disposable, cap-ventilated bottles are available and the 250 ml bottle (working volume 50–100 ml) is widely used [101]. Unfortunately, gas transfer rates for these bottles have not been reported.

Based on the confidence gained with the TubeSpin technology, orbital shaking as a mixing principle for cylindrical vessels has now been scaled up to the 250 l scale of operation (introduced commercially since 2010). In our laboratories, we also use a prototype 2500 l scale reactor [8]. For all scales, very high gas transfer rates and short mixing times were found, allowing high-density cell culture without the necessity to use pure oxygen (a requirement for stirred tank bioreactors). Even 'non-controlled' bioreactors perform extremely well [9]. Oxygen-probes have become obsolete, since the oxygen tension in the liquid can be kept higher than 30% (relative to air). Air-flushing into the headspace of the bioreactor, together with the liquid-induced acceleration of the headspace gas, assures a high driving force for oxygen as a nutrient. Simultaneously, efficient stripping of $CO_2$ from the culture occurs [10]. Successful production runs at the 200 l scale have been carried out without the use of any base additions – essentially reproducing the achievements in TubeSpin bioreactors.

TubeSpin bioreactors, together with shaken Erlenmeyer flasks, have now become a widely used tool for media screening efforts and for process development work with animal cells. We have observed in TubeSpin bioreactors 3–5 g/l for some secreted proteins in fed-batch processes, matching or exceeding the yields reported from fully controlled, stirred steel-based or disposable bioreactors. However, the transfer of processes from the non-controlled TubeSpin reactor to the controlled, stirred tank system faces new challenges. Frequently, yields and cell culture performance in general are disappointing when try-

ing to apply similar process conditions. We speculate that this is due, at least in part, to the longer mixing times, lower gas transfers, and higher shear stresses in stirred tanks. With poor gas transfer for the removal of $CO_2$, the addition of bases for pH stabilization has potentially strong adverse impacts (bulks of liquid with low pH values are floating within a stirred tank for minutes and are surely damaging or killing cells). In addition, pure oxygen, introduced into the gas flow, generates locally in the liquid 'supersaturated' environments five-times higher than would be ever seen in nature. It is a fair assumption that such high concentrations, even on short, but repeated exposure, cannot be cell-friendly.

Overall, cell culture technology for suspension cultures in industrial applications has now been liberated from certain pre-conceived concerns of fragility, and with this an array of new reactor concepts are being explored that are simpler and more robust, including a large diversity of new 'disposable' bioreactor concepts. The stirred tank will survive – however, some of its inherent limitations have been recognized and should be addressed. Interesting is the possibility to run production cultures without fixed pH and $O_2$ values and to have the biology of the cell determine the trends of these parameters.

### Financial & competing interests disclosure

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