Design of Experiments Helps Optimize Cell Culture Bioproduction System

Serum-free culture media used in bioprocessing can typically have 60 – 90 components at differing concentrations for a single cell. Futhermore, media used to grow different cell lines for bioprocessing applications may each require unique optimal chemical formulations. Adding to the complexity, the optimal process conditions such as pH and stirring rate may also differ from cell line to cell line depending on the unique characteristics of process performance. To tackle all these variables Invitrogen Corporation (Carlsbad, California) utilizes design of experiments (DOE) methods, which reveal the complicated array of multi-factor interactions involved in bioprocess development. Some of the experiments conducted include the use of high throughput tools such as a robotically-controlled micro-bioreactor system capable of conducting hundreds of simultaneous bioreactor experiments.

"We know that a sound DOE strategy combined with the right tools can be used to identify truly optimal formulations and operating conditions to maximize product output," said Steven Peppers, Principal Scientist at Invitrogen. "The DOE method greatly reduces the number of experiments and time needed to optimize a large numbers of variables. Some of our DOE methods present the results in the form of a response surface map, making it possible for researchers to quickly zero in on the optimal value of each factor. DOE also provides valuable understanding of the design space, the possible operating intervals of all factors during final application. This information, which could not easily be obtained previously, will help process developers establish thresholds levels for robust manufacturing of their product."

Challenge of designing animal origin free cell culture media

Most biotechnology companies are moving away from media containing undefined components, such as bovine serum and plant hydrolysates. They prefer media free of them for three major reasons. First, sera and other components of animal origin have the potential to contaminate the final product with adventitious agents such as viruses and prions. Second, plant hydrolysates in media sometimes result in high lot-to-lot variation of the selected protein production. Lastly, they can introduce other contaminants that will need to be eliminated during protein purification. While some

suppliers of plant hydrolysates have been taking steps to improve their manufacturing process and reduce this variability, Invitrogen has chosen to develop a portfolio of cell culture products completely free of serum, hydrolysates and animal-origin materials. This portfolio has been attained by balancing the concentrations of other media components and by substituting plant, microbial or synthetic chemicals for animal-derived raw materials. To ensure that bioreactors operate at maximum efficiency and consistency, it is often necessary to optimize the medium to the nutritional requirements of a particular cell line; hence the need for statistically designed experiments.

The traditional approach to optimizing media and operating conditions involves running one-factor-at-a-time (OFAT) experiments. The concentration of a component or the value of a processing condition is varied while all other components and conditions are held constant. One problem with this approach is that there are too many factors to optimize the media fully for a particular cell line. Running batch or fed-batch experiments might tie up a 5-liter research bioreactor for two weeks. If each of 10 factors were tested sequentially, that could require several months! The other problem is that the OFAT approach does not account for interactions among components and conditions. This means that even if all the OFAT experiments were completed, the combined results would miss optimal performance.

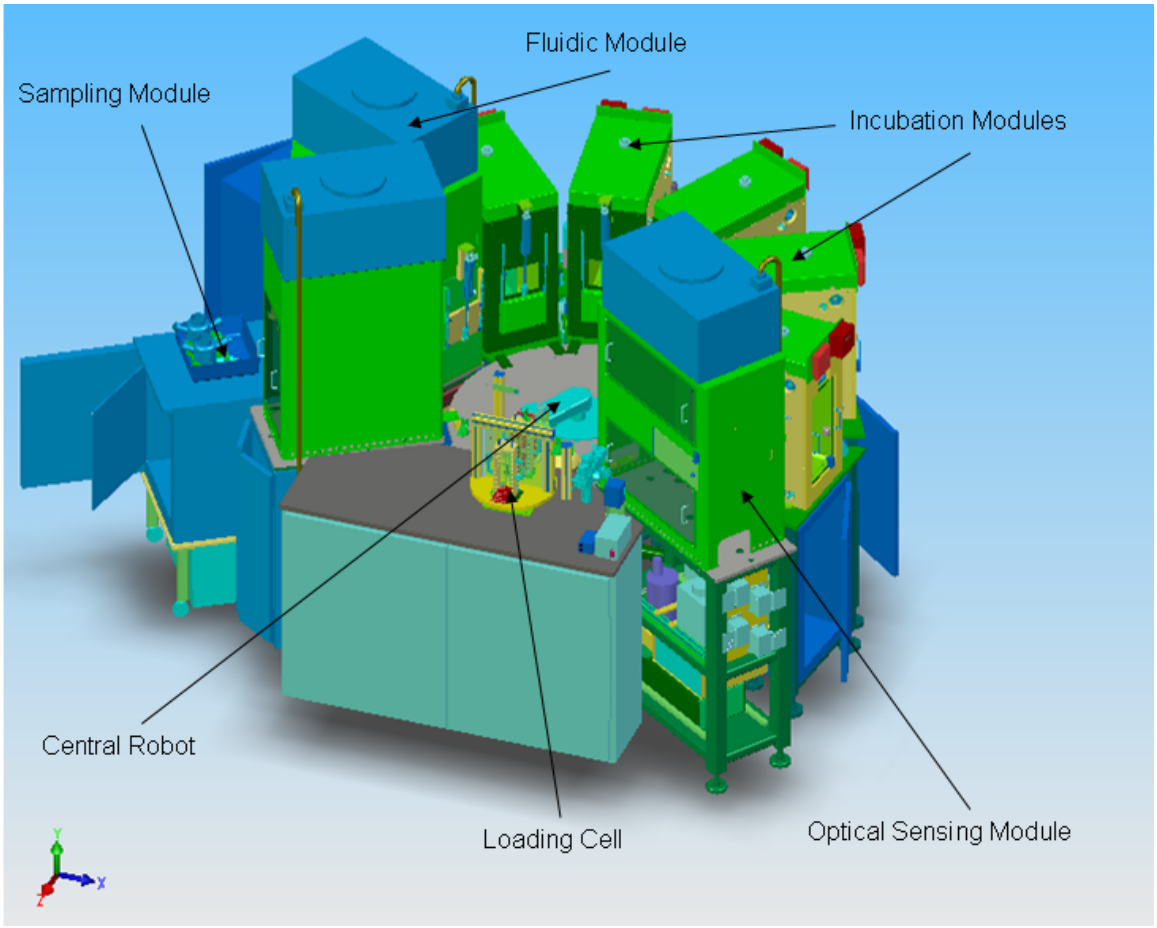

Figure 1: BioProcessors' SimCell™ system

Microbioreactor and DOE enable new methodology

Invitrogen applies DOE methods to both reduce the number of runs required as well as to increase the experimental space evaluated. These methods account for multiple variables and their interactions by testing all factors simultaneously in balanced statistical designs. To reduce the number of runs required, Invitrogen is using instruments such as BioProcessors' SimCell™ high-throughput cell-culture system. This process development system makes it possible to grow animal cell cultures in microbioreactor chambers of approximately 700 µl working volume. There are 6 chambers per microbioreactor array (MBA), which has the same footprint as a standard 96-well plate. As the MBAs rotate around a spindle inside the incubator, an air bubble within each chamber traverses the distance around the perimeter to provide continual stirring of the suspended cell culture. On-line measurements of cell density (optical refraction), pH and dissolved oxygen enable daily monitoring. The pH within each chamber is controlled to a set

point by the automatic addition of a basic solution after a pH reading dips below a programmed threshold. Gas concentrations (CO_2 and O_2), temperature and rotation speed within each incubator are also adjustable. Various feed solutions may be programmed for introduction into the chambers during specific run cycles. Chamber suspensions may also be sampled into 96-well plates throughout the run to enable off-line measurements, such as viability and protein production.

"The type of experiments we need for optimizing media are complex and would be impractical to manually design and analyze," Peppers said. "Fortunately, software is available that greatly reduces the time required for experimental design and results analysis. We use Design-Expert® software (from Stat-Ease, Inc., Minneapolis, Minnesota), because it offers a wide choice of experimental designs and provides an intuitive user interface. We often use fractional factorials and minimum-run designs to identify key effects and interactions. Sometimes we augment these to central composite designs (CCD) because these enable response surface maps that help us understand the results more fully. For example, if the response continues to rise at one of the edges of our design space, this indicates that the optimum probably lies outside the tested range of factors. A steep curve around the optimum indicates that the response is very sensitive to one or more factors. On the other hand, a flat surface or shallow curvature near the optimum indicates that the response is more robust and less likely to be affected by changes in those particular factors."

Starting Media and pH Conditions					**Feed Additions, Day 4**		
pH	**Osmo Target**	**Glucose**	**Glutamine**	**Pluronic**	**Glucose Feed**	**Glutamate**	**Aspartate**
6.9	**246.2**	**.64**	**.32**	**.8**	**0**	**0**	**0**
6.9	270	2	1	.8	0	0	0
7.1	**305**	**4**	**2**	**1.3**	**2**	**2**	**0.1665**
7.3	340	6	3	1.8	4	4	0.3330
7.3	**363.8**	**7.36**	**3.68**	**2.14**	**5.36**	**5.36**	**0.4462**

Figure 2: Factor level definitions for central composite design (CCD) experiment

Central composite design experiment

Peppers and his associates recently performed a designed experiment that confirms the ability of the microbioreactor and DOE to optimize complex

cell-culture media. He used a fractional CCD with eight factors at five levels as shown in Figure 2. The fractional CCD provided by Design-Expert software required 192 runs (a full 5-level factorial would have required 390,625 runs!). These runs were all performed in parallel in the microbioreactor in a total of 8 days. Peppers entered the results into Design-Expert and the software provided a complete statistical analysis of the results.

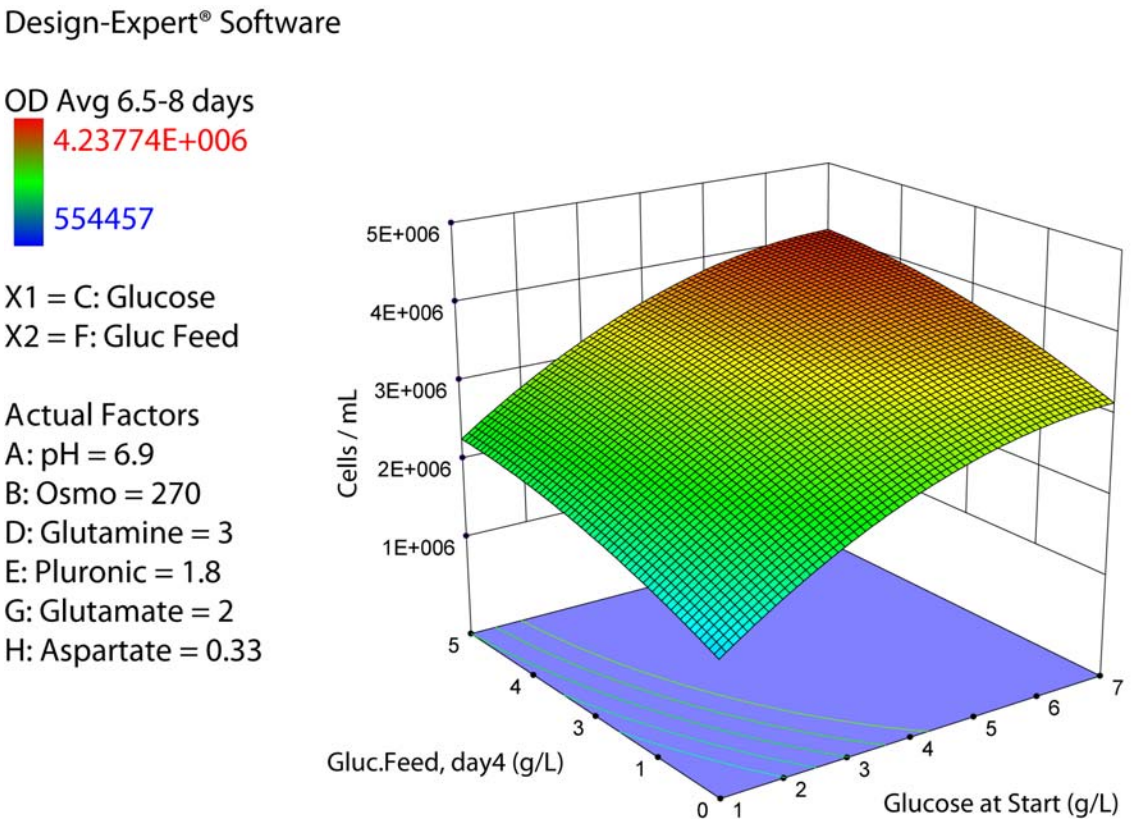

Figure 3: 3D surface plot of cell density in response to initial and feed glucose concentrations

The response surface map shown in Figure 3 confirmed the understanding that Invitrogen researchers had developed over years of OFAT experiments on how glucose enables growth. The vertical axis depicts the model-generated average cell densities at late phase growth (days 6-8). The axis moving off the origin to the right represents increasing amounts of glucose in the initial media. The axis moving off the origin to the left represents increasing amounts of glucose added to the bioreactor at day 4 of incubation.

This response surface is lowest when both glucose at start and glucose feed are minimal and highest when both of these are maximal. The surface

curvature illustrates that the predicted cell density is much less sensitive to changes in glucose near the highest tested glucose concentrations than near the minimum glucose concentrations. "These results are aligned with what we had expected, which reinforces our confidence in the equipment and experimental design," Peppers said.

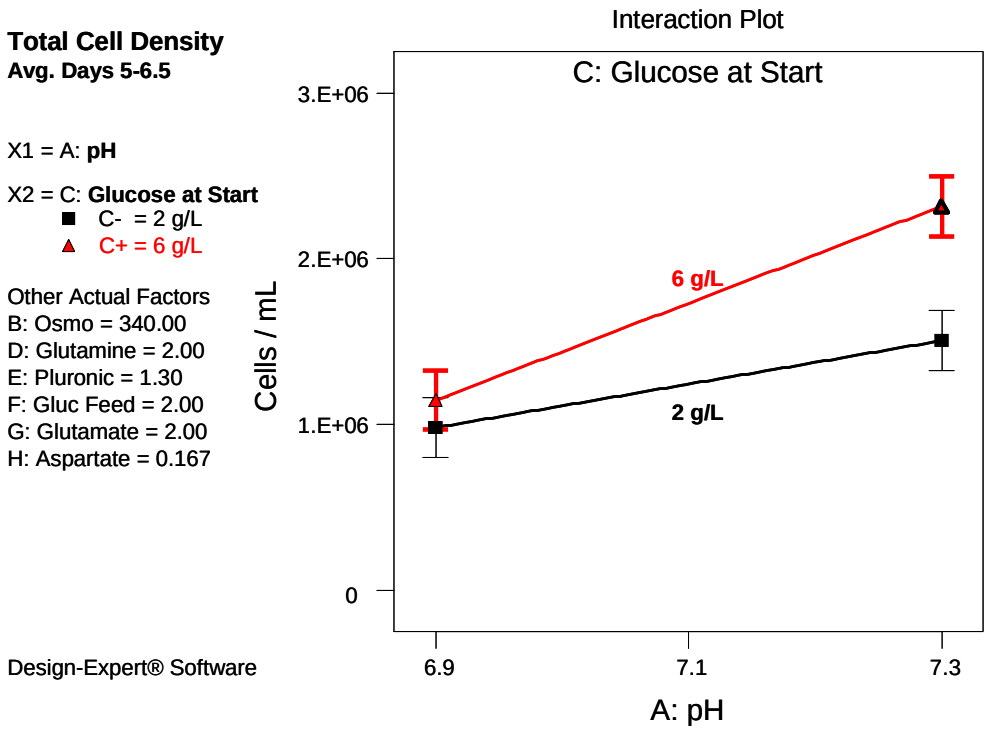

Figure 4: Interaction of Initial Glucose and pH during mid-to-late log phase growth

Invitrogen researchers generated additional statistical output to investigate interactions between two factors. Figure 4 depicts the effects of pH on the average total cell density in mid-to-late log phase growth (days 5 to 6.5) for cultures begun with different initial glucose concentrations. When initial glucose concentration was low, changing the pH had very little impact on cell growth. When initial glucose was high, the cultures at pH 7.3 grew to considerably higher cell density than at 6.9. The error bars represent ± ½ least significant difference (LSD) at α=0.05 (95% confidence); non-overlapping bars indicate a significant difference between means. "The ability to understand these types of multivariable interactions, which are not

visible in OFAT experiments, is a key advantage of the DOE method," Peppers said.

Selected Outcome	Set to	Weight
Cells/mL, days 5-6.5	Maximum	3
Cells/mL, days 6.5-8	Maximum	3
Prod'n of tPA, day 8	Maximum	5

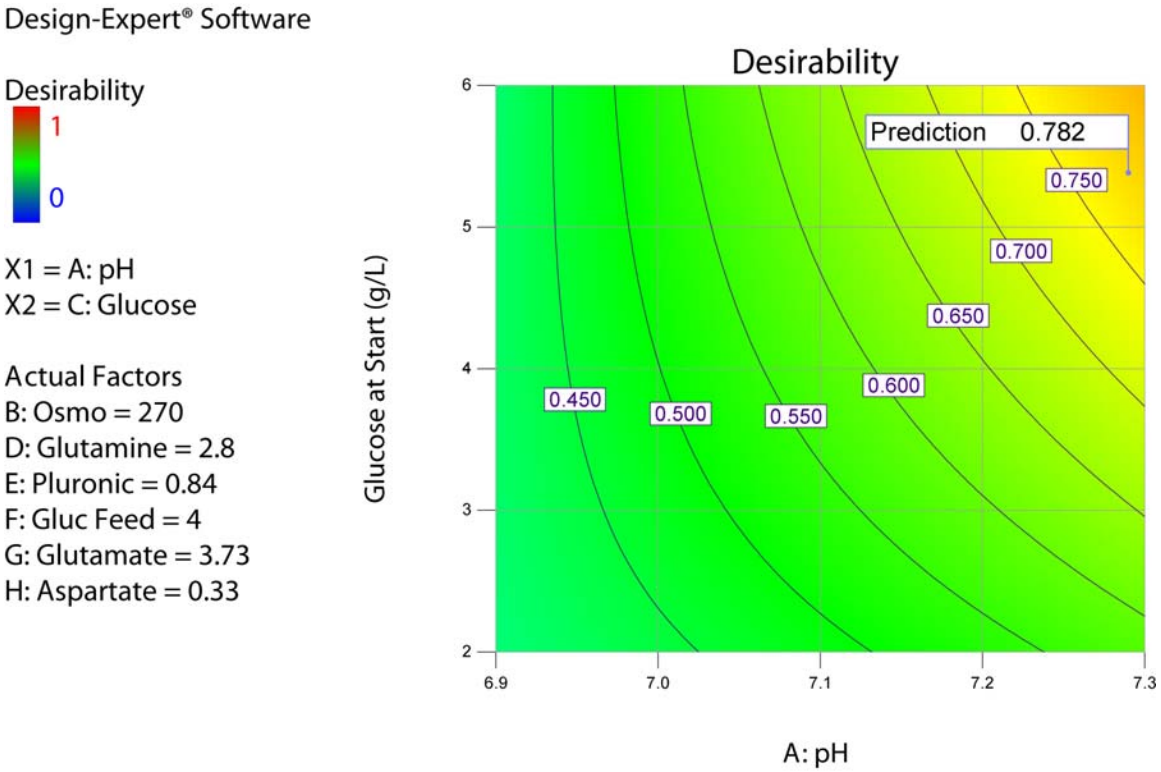

Figure 5: Contour plot of desirability function to optimize cell density and productivity

Optimizing cell density and productivity

Figure 5 shows another way of looking at the results of the experiment. Peppers defined a desirability function that includes cell densities in mid-late and late growth phase of the experiment and production of tissue plasminogen activator (tPA). The computational weights associated with these three parameter inputs are 3, 3 and 5, respectively. The range of each factor was limited to the core region of the CCD. Based on the experimental results, the software identified a set of optimal values for each factor (the left side of Figure 5) resulting in a desirability value of 0.819 on a scale of 0 to 1.

“This experiment demonstrates how a robotically controlled microbioreactor system can be combined with DOE methods to optimize cell-culture media and feeding strategies” Peppers concluded. “This new process is rich in information and provides a solid understanding of the most influential factors affecting performance of specific cell lines. As we integrate these techniques into our media design and manufacturing process, we will be able to provide our customers with media and feeds that greatly enhance performance in the bioreactor.”

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