Chinese hamster ovary (CHO) cells commonly are used to produce recombinant proteins such as monoclonal antibodies (MAbs) for research, diagnostic, and therapeutic purposes. Culture processes typically rely on a fed-batch approach in which a basal medium enables initial cell growth. Concentrated feeds are used to prevent nutrient depletion, thereby extending culture duration and improving cell growth, viability, and protein titer.

A neutral pH feed is desirable because culture pH should remain stable after feedings. The extremely low solubility of L-tyrosine and the low stability of L-cysteine at neutral pH have necessitated processes that leverage a slight acidic main feed and a separate alkaline feed containing these essential amino acids. Use of separate feeds at different pH levels creates the need for complex control strategies to minimize pH spikes during feed additions. In bioreactors, the alkaline feed can be added slowly while pH is monitored continuously and proportional-integral-derivative (PID) settings are adjusted to reduce CO₂ sparging response to spikes in pH. In large-scale manufacturing, even with slow addition of feeds, precipitation is likely to occur after contact with neutral pH media, especially when residual foam is present.

Here we summarize results of in-depth studies that defined a simplified approach to fed-batch processes in large-scale manufacturing using a highly soluble phosphotyrosine sodium salt and a new derivative, S-sulfocysteine, in neutral pH, highly concentrated feeds (Figure 1) (1, 2).

### Tyrosine

Depletion of L-tyrosine in fed-batch processes has been correlated with a decrease in specific productivity and protein sequence variants (3, 4). L-tyrosine has an extremely low solubility and is difficult to dissolve when concentrations of tyrosine disodium salts >1 g/L are used. Such high concentrations further increase the risk of media instability, mainly through coprecipitation of other amino acids. That can lead to insufficient supply of nutrients and less-than-robust processes (data not published).

To address the challenge presented by tyrosine and eliminate the need for a separate alkaline feed, we evaluated the use of a phosphotyrosine disodium salt (PTyr2Na⁺) as a source of L-tyrosine in chemically defined, neutral pH feeds for CHO fed-batch processes. Table 1 shows our key results.

### Cysteine

Low stability of cysteine results from dimerization and precipitation of L-cystine at neutral pH and 25 °C.
also is critical for fed-batch processes. As with l-tyrosine, an alkaline feed containing l-cysteine is used commonly, presenting similar challenges with precipitation and pH spikes. Because l-cysteine is not stable at neutral pH, we tested a new derivative, S-sulfocysteine sodium salt (SSC) in neutral pH feeds with a CHO cell line expressing a human MAb. In SSC, the highly reactive thiol group of cysteine is blocked by a sulfate group, which lowers the molecule’s reactive potential toward metals such as copper and iron and oxidation. Table 2 summarizes key results.

Figure 2 compares the single-feed strategy using PTyr2Na⁺ and SSC to a fed-batch process using the classical separate cys/tyr feed at pH 11. Results show a longer culture duration and a doubling of specific productivity with the single-feed strategy.

We also explored possible mechanisms leading to the increased titer when using SSC. Intracellular labeling with carboxy-H2DCFDA demonstrated a decrease in reactive oxygen species (ROS) in the cells obtained in the SSC-containing process, pointing out to antioxidative mechanisms.

We performed microarrays on mRNA of CHO cells extracted at different time points in the fed-batch process. The gene showing the highest upregulation in the SSC condition compared with the control coded for SOD-2. Superoxide dismutases catalyze conversion of superoxide radicals to H₂O₂ and O₂ and are known to decrease intracellular ROS levels (5). The enzymes may be partly responsible for the lower intracellular oxidative potential observed in CHO cells.

Cysteine is a key sulphur source and is the limiting factor for synthesis of glutathione (GSH), the main intracellular antioxidant. Although microarray studies showed no change in expression of enzymes involved in GSH synthesis, CHO cells cultured in the presence of SSC had an increased pool of total glutathione. That may help explain their longer culture duration as a result of several described mechanisms (6–8). The higher intracellular free glutathione pool may be linked to increased protein folding in the endoplasmic reticulum of those cells, where an oxidizing environment is necessary to add disulfide bonds into the protein (9). That can result in a higher secretory rate — and ultimately, a higher titer.

Finally, one main advantage of SSC is that the highly reactive thiol group of cysteine is protected, thus limiting the interaction of the molecule with other cell culture media components such as metals. SSC also is less susceptible to oxidation than cysteine. This contrasts with the most commonly used cysteine derivatives or prodrugs such as GSH, cysteamine, or N-acetylcyesteine, in which

| Table 1: Evaluation of phosphotyrosine disodium salt (PTyr2Na⁺) as a source of l-tyrosine in chemically defined, neutral pH feeds for CHO fed-batch processes |
| Evaluation | Results |
| Solubility and stability of phosphotyrosine disodium salt in chemically defined media and feeds | In water, the solubility of PTyr2Na⁺ was evaluated at 53 g/L, more than 100-fold higher than the solubility of l-tyrosine (0.38 g/L). In concentrated feeds, PTyr2Na⁺ exhibited a solubility of 70 g/L by contrast with l-tyrosine, which was not soluble and to l-tyrosine disodium salt for which the maximum solubility was <1 g/L. No degradation or precipitation of phosphotyrosine was observed during the first six months in liquid feed, and no free tyrosine generated from potential oxidation reactions was detected. |
| Effect of phosphotyrosine in fed-batch processes and optimization of tyrosine concentration | For fed-batch processes, adjustment of the concentration of tyrosine in the medium and of PTyr2Na⁺ in the feed is mandatory to achieve an optimal result. With the CHO cell line, optimized growth and specific productivity were obtained with 2.5 mM tyrosine in the medium and 30 mM PTyr2Na⁺ in the feed. |
| Scale-up in 1.2-L bioreactors | No significant difference in performance was detected between the control condition, in which l-tyrosine was added at pH 11, and the condition in which PTyr2Na⁺ was added into the main feed (equimolar end concentrations for both). Both viable cell densities and the final titer were very similar (1.9 and 2.1 g/L, respectively, at the end of the 17-day process). Specific productivity was 14.5 pg IgG/cell/day for both conditions. Use of chemically modified PTyr2Na⁺ in concentrated feeds did not result in a detectable modification in the amino acid sequence or the glycosylation profile of the monoclonal antibody. |

| Table 2: Evaluation of S-sulfocysteine sodium salt as a source of l-cysteine in chemically defined, neutral pH feeds for CHO fed-batch processes |
| Evaluation | Results |
| Solubility and stability of S-sulfocysteine sodium salt in water and chemically defined media | SSC was soluble up to 1.3 and 2.3 M in water at room temperature and 37 °C, respectively, and 50 mM SSC were soluble in the pH 7.0 feed. In water, SSC was stable at acidic (pH 3.0) and neutral pH (pH 7.0), whereas a spontaneous dissociation leading to cysteine release and cystine formation was observed at alkaline pH (pH 11.0). There was no visible precipitation or change in color during three months storage in neutral pH feed (pH 7.0) at 15 mM SSC (target concentration for cell culture experiments). There was no significant decrease of the SSC concentration in the feed stored at 4 °C or room temperature protected from light. |
| Use of S-sulfocysteine in batch experiments | Cultivation of a CHO suspension clone with 1.5 mM SSC showed a similar maximum viable cell density of about 150 × 10⁶ cells/mL and a prolonged viability maintained over 90% at day 7 compared with control condition containing 1.5 mM l-cysteine. |
| Dose response of S-sulfocysteine in spin tube fed-batch process | Maximum viable cell density of all tested SSC concentrations were comparable with the control (130 × 10⁶ cells/mL). Viability was maintained >50% for two additional days for SSC concentrations ≥5 mM. Significantly higher final titer were measured with SSC concentrations ranging from 5 to 15 mM compared with the control. 78% higher final titer were obtained with 15 mM SSC, resulting in a 1.7-fold increase of the mean specific productivity. |
| Use of S-sulfocysteine in 1.2-L fed-batch bioreactor process. For fed-batch experiments, we combined SSC with PTyr2Na⁺ in a single neutral pH feed. | Comparable maximum viable cell density of 150 × 10⁶ cells/mL were observed in the control and 15 mM SSC condition. Prolonged viabilities were detected when using the l-cysteine derivative. Glycosylation and charge variants were monitored at day 12 (viability >90%). The glycosylation pattern obtained in both the control and SSC conditions showed no significant change in the main glycoforms; minor forms were also similar. |
the highly reactive thiol group is free to interact with other media components.

**ADVANTAGES**

MAB manufacturing depends on high-concentration nutrient feeds, which can lead to high titers and sustain specific productivity throughout the culture. Use of phosphotyrosine disodium salt and S-sulfocysteine sodium salt as components of chemically defined, neutral pH, highly concentrated feeds provides an alternative to the commonly used separate cys/tyr alkaline feed. The advantage of the approach summarized here is that it can be incorporated into existing strategies to simplify fed-batch processes in large-scale manufacturing and improve overall process performance.

**REFERENCES**


Joerg von Hagen is R&D director, Caroline Hecklau is a PhD student, Ronja Seibel is a scientist, Sascha Pering is a PhD student, Alisa Schnellbaecher is a scientist, Maria Wehsling is a scientist, Thomas Eichhorn is a scientist, and corresponding author Aline Zimmer is head of R&D, advanced cell culture technology, all at Merck KGaA, Darmstadt, Germany; aline.zimmer@emdmillipore.com. Frankfurter Strasse 250, 64293 Darmstadt, Germany.

For reprints, contact Rhonda Brown of Foster Printing Service, rhondab@fosterprinting.com, 1-866-879-9144 x194.