

# The Development of a Bio-inspired, Chemically Defined Media Supplement for Cell Culture

Justin Oliver\*, Kirti Chaturvedi\*, Damon Barbacci, Cindy Hunt, and Elizabeth Dodson  
*BD Biosciences – Advanced Bioprocessing, Sparks, MD*

## ABSTRACT

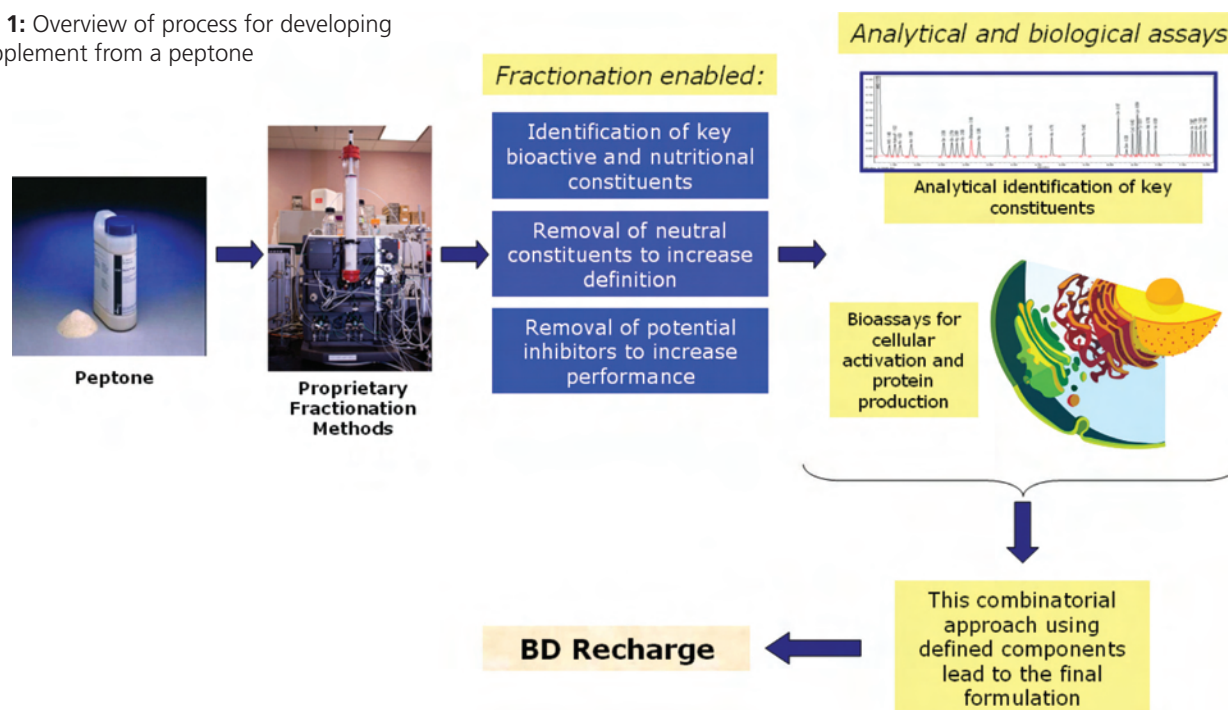
Peptones have a long history of use in cell culture media. However, chemically defined alternatives are often desired for bioprocess applications. Using proprietary chemical separation and analysis methods on peptones has enabled BD to gain a greater understanding of the functional components; this knowledge has inspired the formulation of a new chemically-defined (CD) cell culture supplement, BD Recharge™. Optimization of the components through Design of Experiments has resulted in a formulation which is functionally comparable to the starting peptone. In addition to being chemically defined, the composition of BD Recharge is fully animal-free and protein-free, distinct advantages over peptones. In testing on Chinese hamster ovary (CHO) cells, growth and protein yields are equivalent or superior to that seen with peptone supplementation. Moreover, an assay of mAb protein quality is also found to be equivalent. These attributes suggest that BD Recharge is an ideal substitution for peptones, affording greater lot-to-lot consistency while maintaining protein quality and production.

## INTRODUCTION

Peptones are hydrolysates of plant or animal origin. Consisting of complex mixtures of amino acids, polypeptides, nucleic acids, vitamins, and other nutrients, peptones have long been used successfully as components of microbiological media, and somewhat more recently, as supplements in media for eukaryotic cells. Animal-free hydrolysates mitigate the safety risk of using either serum or animal-origin hydrolysates for improving cell culture performance; however, animal-free hydrolysates are still a biological product with some inherent variability. This variability may further impact the existing variability that is present in cell-based bioprocess methods. In recent years an increasing emphasis has been placed on growing cells in animal-free and chemically-defined systems for generation of biopharmaceuticals. This has created a demand for the development of cell culture media supplements that are equal in performance to peptones but have greater certainty of composition.

This demand prompted BD's efforts to identify the functional components of peptones and to derive a chemically defined supplement based on this information.

**Figure 1:** Overview of process for developing CD supplement from a peptone



\*These authors contributed equally to this work.

As a first step toward a peptone-inspired, chemically defined supplement, separation processes were used to provide hydrolysate sub-fractions of reduced complexity. Bioassays on several cell lines provided a read-out on the positive, negative, or neutral fractions. Chemical analysis and identification allowed the transition to testing of defined components, singly and in groups.

Statistical design of experiment (DOE) approaches were used to arrive at an optimized supplement formulation with equivalent or superior performance when compared to peptones in cell culture. These steps are outlined in Figure 1. This paper describes the development of a CD supplement using this approach.

## MATERIALS AND METHODS

### Fractionation

Proprietary separation processes were used to provide peptone sub-fractions of reduced complexity. Yeast extract was the peptone used for this study. Yeast extract fractions were generated using several chromatographic and related methods of chemical separation. The fractionation and work-up methods were optimized to allow minimal carryover of salts and other contaminants from the separation method into the final fractionated material for testing. In cases where carryover was a concern, salt controls were included in the workup procedure and the subsequent testing to account for any effects.

### Bioassay

Bioactivity of peptone fractions, chemically-defined components and chemically-defined supplements was tested using several CHO host cell lines, for example, CHO-K1, CHO DG44, and GS-CHO. These CHO cells stably expressed monoclonal IgG antibodies (mAb's), enzymes (e.g., *Metridia* luciferase), or other, non-enzymatic proteins. Cell culture assays were carried out in 125mL shaker flasks, seeded at  $2 \times 10^5$  cells/mL, or in 50mL TPP TubeSpin® tubes, seeded at  $0.5 \times 10^5$  cells/mL, in a shaking, humidified CO<sub>2</sub> incubator set at 5% CO<sub>2</sub> saturation and 125 RPM. Cultures were sampled for assessment of cell growth and protein production. The quantification of cell number and viability was performed using a Beckman Vi-Cell®. Protein production was measured using Protein A biosensors for IgG producing lines, on a ForteBio Octet® system. An enzyme activity assay was used

for the *Metridia* luciferase-producing line (e.g., measurement of luciferase bioluminescence at 480nm with a Perkin-Elmer Envision® platereader); ELISA was used for other recombinant protein producing cell lines. The chemically-defined base media used varied according to the cell line.

### Biochemical Component Analysis and Identification

Chemical analysis performed on the peptone and its fractions included the following: free and total amino acid analysis using the Waters AccQTag™ system and Waters ACQUITY UPLC®; vitamin, nucleic acid, and lipids analysis using an Agilent 6520 Accurate-Mass Q-TOF LC/MS or 6890N GC/MS; ICP-MS elemental analysis using an Agilent 7500cx ICP-MS; molecular weight distribution analysis by size-exclusion chromatography using a Waters Alliance® HPLC; and carbohydrate determination using a colorimetric method. In addition, some fractions were analyzed with high-resolution LC/MS-MS for peptide analysis and sequencing or for small-molecule identification.

### Protein Quality Analysis

An HPLC cation-exchange method for separating and detecting protein charge variants was used to analyze monoclonal antibodies produced under varying conditions. mAbs were purified from culture media using a protein A column (GE HiTrap™) and eluted from a weak cation-exchange column (Dionex ProPac® WCX-10), using a NaCl gradient, on an Agilent 1100 HPLC using UV detection at 280nm.

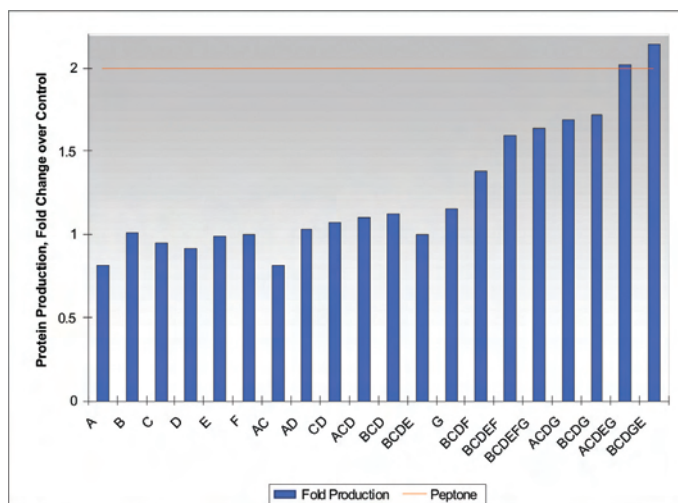
## RESULTS AND CONCLUSIONS

### Identification of Peptone Functional Components

Through iterative rounds of yeast extract separation, fractions and sub-fractions of the peptone were generated. These fractions were initially screened for their growth and recombinant protein production performance using representative CHO lines in TubeSpin® cultures, and the activity of fractions that boosted production over media control (positive fractions) was confirmed in shaker flasks. In addition, some fractions had a deleterious effect on growth and/or production (negative fractions). Chemical analysis was performed on both types of fractions. From the positive fractions, a set of components was identified that correlated with improved growth and production performance in cell culture. Inhibitory components were also identified from negative fractions. Although present in the native yeast extract, these negative factors appeared not to have a detrimental impact on peptone performance in the context of the whole peptone.

The effect on protein production and growth of combinations of positive components was determined in two CHO cell lines. Some of these components or component groups were found to be essentially nutritional in nature, supporting the viability of the cells, while having minimal effect on recombinant protein production; other components or component groups, however, elicited increases in protein titer and so were termed bioactives

**Figure 2:** Effect of yeast extract-derived defined components or component groups, singly and in combination. Data are presented as fold change in protein production of a mAb-producing CHO line over negative control (medium only). The orange line represents the fold change of the yeast extract positive control.

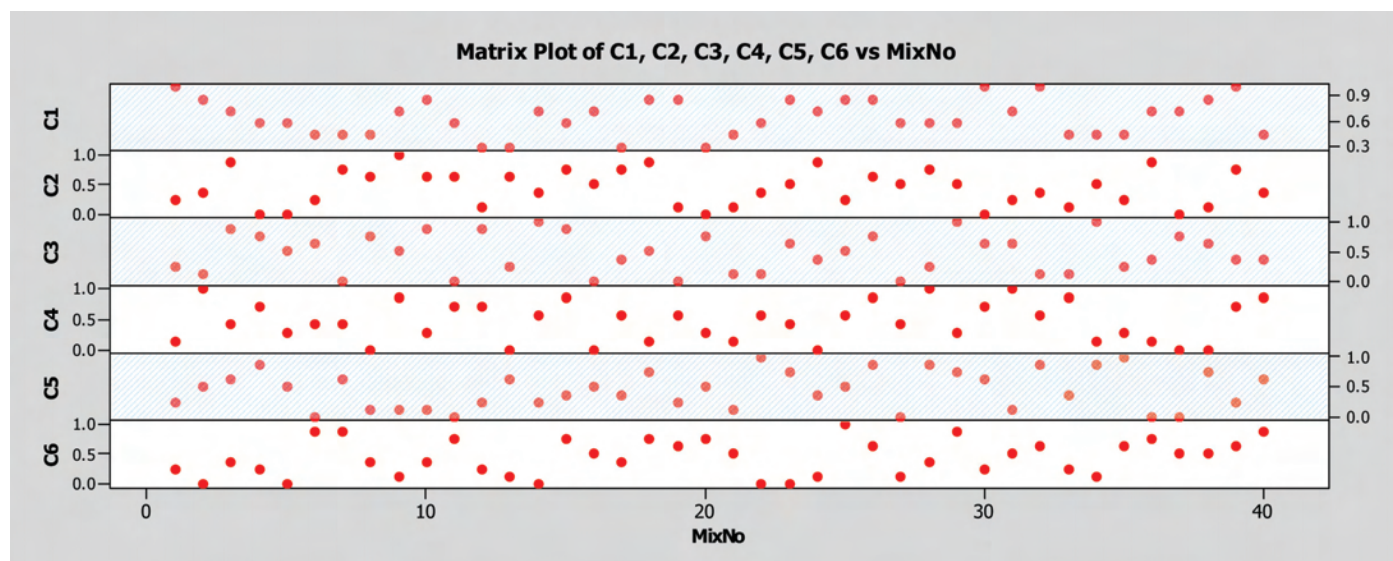


(data not shown). Figure 2 shows protein production data from a mAb-producing CHO line in a representative component mixing experiment. Seven components or groups of components of the same class were titrated, either alone or in combinations, into the base media, and the maximal protein production from each combination was measured. All of these components or component groups (A-G) showed negligible effects on protein production when used alone, or in combinations of two or three, to supplement the culture medium. However, when used in combinations of four to six, including nutritional and bioactive, significant increases in protein production were observed. Only certain combinations of nutritional and bioactive components gave an increase in protein production comparable to that seen with yeast extract positive control. Furthermore, addition of *more* components did not always give optimal results (*e.g.*, the BCDEFG combination). A preliminary CD supplement formulation was developed by combining nutritional and bioactive components.

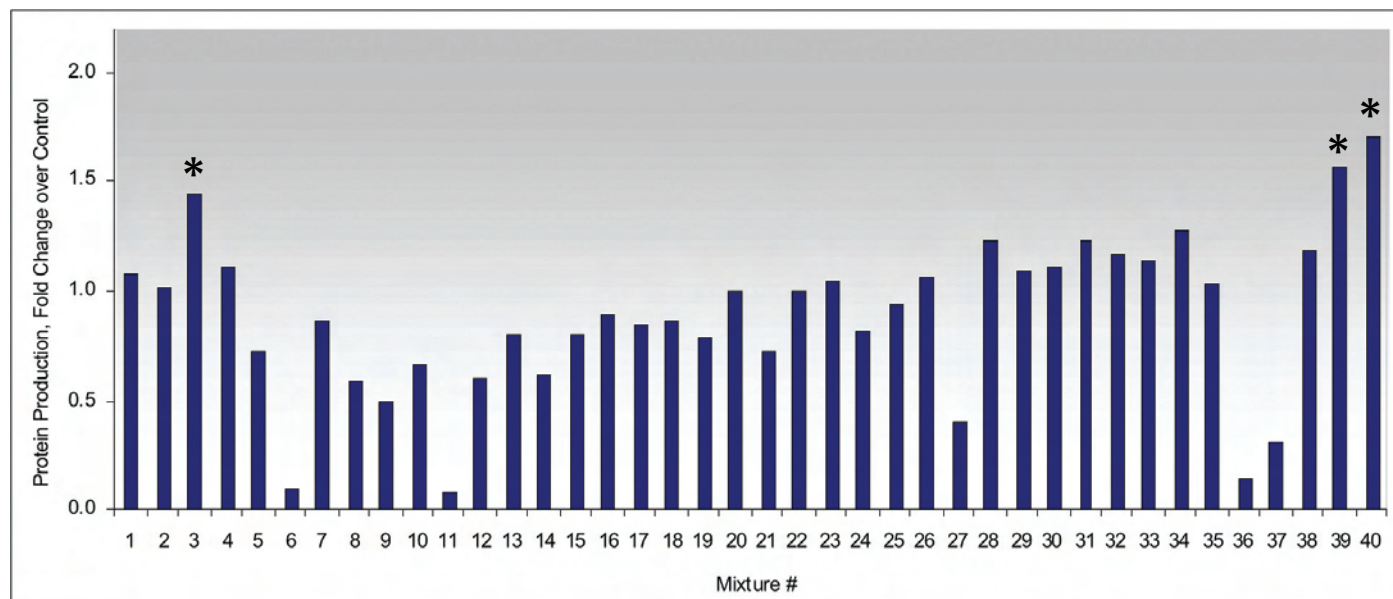
### Optimization of the Formulation

After the generation of a preliminary CD formulation, several fractional factorial screening experiments were carried out to optimize the levels of components in the mixture. For example, the effects of six bioactive components were examined using the space-filling DOE design shown in Figure 3. A total of 40 formulation mixtures, with varying levels of all six bioactive components in each mixture, were tested in CHO cells producing *Metridia* luciferase. Cell growth and protein titer were assessed for each mixture during culture. Figure 4 shows the protein production levels, in terms of fold change over media-only negative control, of the 40 mixtures on day 12 of the culture. Many of the mixtures showed negligible difference from the negative control. There were certain mixtures that were detrimental to cell growth (data not shown) and showed very low production (mixtures 6, 11 and 36). Three mixtures, 3, 39, and 40, showed a response higher than media-only. The formulations in these three positive-

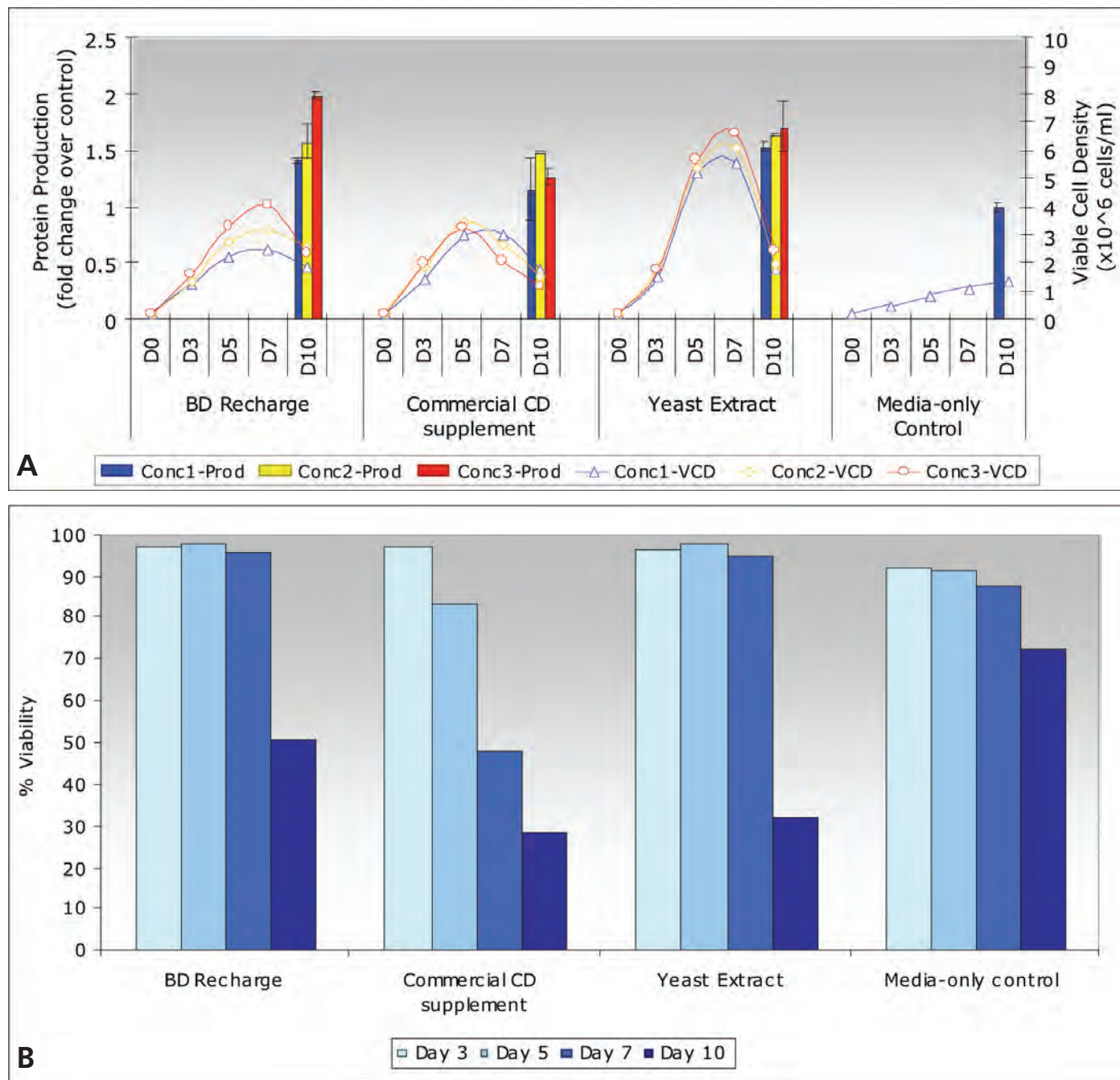
**Figure 3:** Example of Experimental Design for optimization of six bioactive components (C1-C6). The relative component concentration is indicated on the y-axis of each row; the mixture numbers are indicated along the x-axis.



**Figure 4:** Effect of DOE Mixtures shown in Figure 3 on protein production. Data are presented as fold change in protein production of a luciferase-producing CHO line over negative control (medium only) on day 12. Stars indicate mixtures with improved performance.



**Figure 5:** 5A; Performance of BD Recharge vs. a commercial supplement, yeast extract, and media-only control. Supplements were added to base media on day 0 (D0) at three different concentrations for each supplement (Conc1, Conc2, and Conc3). Production is indicated by bars (Prod), viable cell density by lines (VCD); error bars indicate  $\pm 1$  s.d. for duplicate flasks. 5B; Percent cell viability during the experiment for the highest concentration (Conc3) is shown.



responding mixtures were further tested on additional CHO cell lines to select the mixture with the broadest applicability. Multiple rounds of optimization using mixing studies similar to the one described above resulted in the development of the final CD supplement formulation, BD Recharge.

*Performance in Cell Culture*

The performance of BD Recharge was confirmed in various CHO cell lines and compared to peptone positive controls. For all the cell lines tested, the supplement showed performance at least equivalent to the yeast extract control. A representative experiment comparing a titration of BD Recharge, a peptone positive

control (yeast extract), and media-only negative control is shown in Figure 5A. Performance of the supplement was also compared to another commercial supplement. A mAb-producing CHO K1 cell line was tested in batch mode for this study; all conditions used the same CD base medium. BD Recharge was used at 0.43, 0.87, and 1.7 g/L, yeast extract at 1, 2, and 3 g/L, and commercial supplement at 1.8, 3.5, and 7 g/L (in each case the concentrations of supplements are listed in Conc1, Conc2, Conc3 order, see Figure 5A). Pilot titrations had previously shown that these concentrations would be near the optimum for this cell line, with higher amounts giving a productivity plateau or decline. Samples were collected over the course of the experiment for viable cell

density and on the final day of the culture (day 10) for protein production. As shown in Figure 5A, the protein production increase over media-only with BD Recharge at 1.7 g/L was comparable to that with yeast extract at 3 g/L. It is typical that the optimum concentration of BD Recharge is approximately half that of yeast extract. Under the conditions tested, BD Recharge produced a 2-fold increase in protein production over media control at day 10, while the commercial competitor supplement produced a 1.5-fold increase and yeast extract a 1.7-fold increase. In all supplemented conditions, the viable cell density in the batch cultures reached a maximum between days 5 and 7, with the yeast extract maximum at approximately twice that of BD Recharge or the commercial CD supplement. Using this cell line, BD Recharge elicited the same or greater boost in protein production as yeast extract with significantly less cell density. Figure 5B shows that BD Recharge supported improved cell viability over the other supplements; at the highest concentration used, the day 10 viability of the culture was 51% with BD Recharge, while it had declined to 33% with yeast extract and 29% with the commercial supplement. This viability benefit of BD Recharge was seen at all concentrations (data not shown).

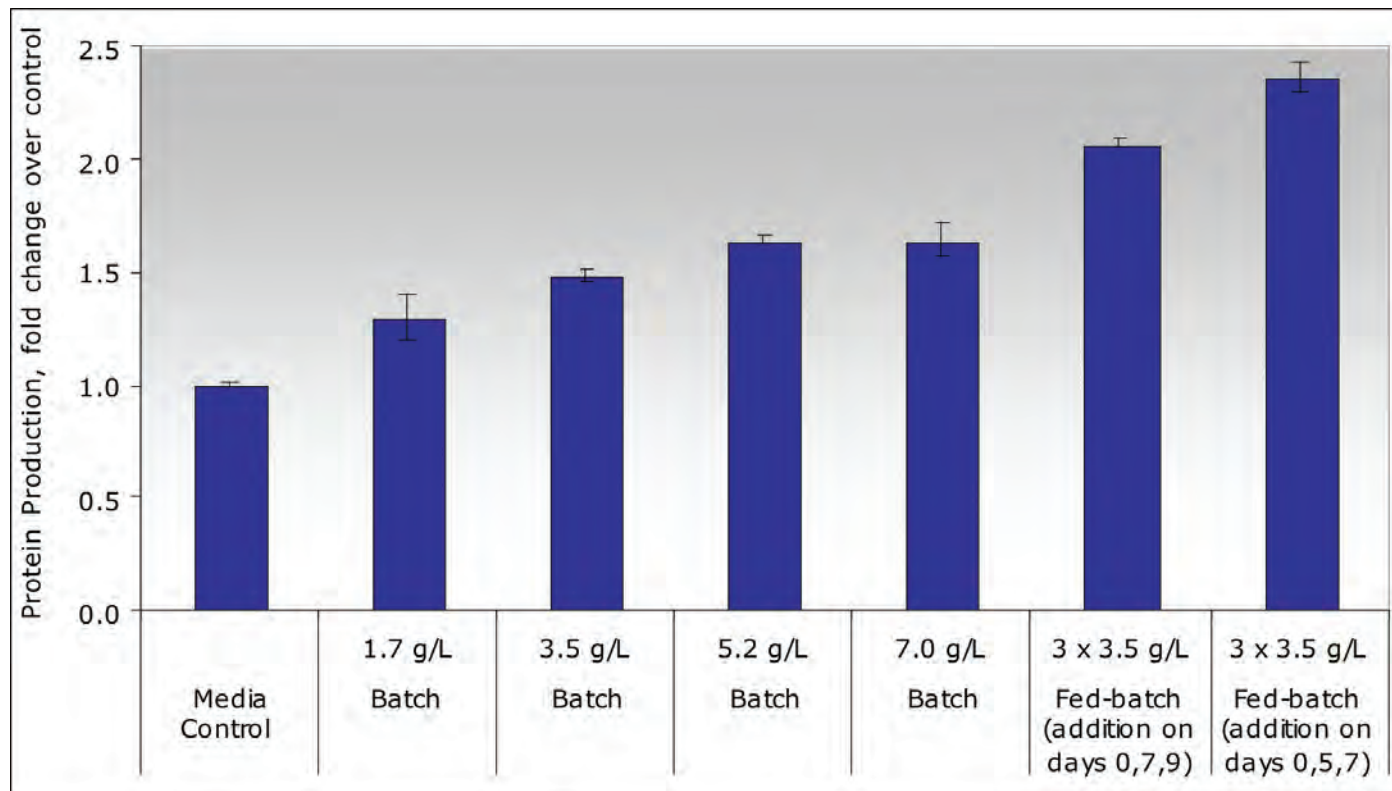
The effectiveness of fed-batch strategies to increase growth and production performance was examined. A shaker-flask study was performed to determine the protein production enhancement over negative-control (un-supplemented) media in a mAb-producing GS-CHO line using a batch or fed-batch approach. For this study, cells were either fed only at the beginning of the culture or batch-fed on selected days of the culture. Samples were collected over the course of the cultures for measurement of viable cell density and protein production. As shown in Figure 6, a concentration-dependent increase in protein production on day 15 was observed

when BD Recharge was fed only at day zero (batch), with the effect reaching a plateau at approximately 5 g/L of supplementation. An improvement in protein production over batch conditions, for this cell line, was seen when the supplement was provided in three feeds during the run, with the best performance shown by feeding on days zero, five, and seven. In addition, the viability of the fed-batch cultures was higher on day 15 over the batch culture conditions (e.g., 69% viability for the fed-batch conditions vs. 59% for the 7.0 g/L batch; data not shown).

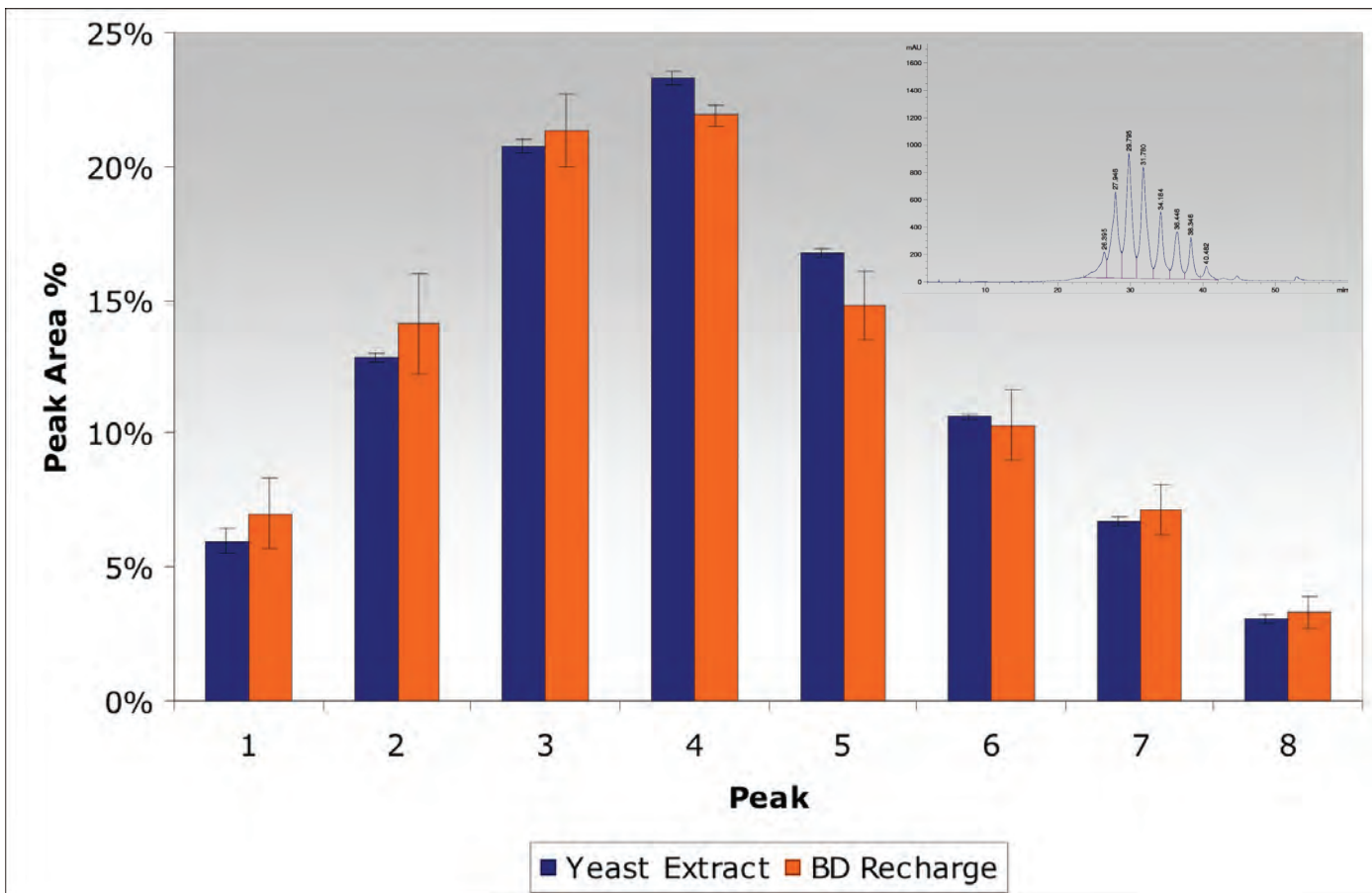
#### Protein Quality

The post-translational modifications (PTMs) observed in biotherapeutics are the result of a complex set of factors, including the media and supplementation used<sup>1</sup>. One commonly used measure of the overall PTM state or protein quality is the variation in charge revealed by cation-exchange chromatography. To examine the comparability of proteins produced with BD Recharge and yeast extract, a mAb-producing CHO line was grown under identical conditions except for supplementation. One culture was fed BD Recharge, the other yeast extract peptone, both in batch. On day 12 of the culture, a media sample was taken from each culture, and the antibody in each was purified using Protein A chromatography. The purified proteins were then passed over a cation-exchange chromatography column to separate charge variants. Figure 7 shows that the areas of the charge-separated peaks were comparable between the two supplementation conditions, indicating that the charged PTMs arising during protein production were roughly indistinguishable whether BD Recharge or peptone was used as the medium supplement.

**Figure 6:** Protein production performance on day 15 using a batch vs. fed-batch approach with BD Recharge. Data are presented as fold change in protein production of a mAb-producing CHO line over negative control (medium only). The level and mode of supplementation (batch vs. fed-batch) are indicated along the x-axis. Error bars indicate  $\pm 1$  s.d. for duplicate flasks.



**Figure 7:** Cation-exchange peak areas in mAb produced using BD Recharge or yeast extract supplementation. The bars represent the areas under the curve of the peaks from the separation. Data were acquired from triplicate injections of two independent biological assays; error bars represent  $\pm 1$  s.d. An example chromatogram is shown in inset.

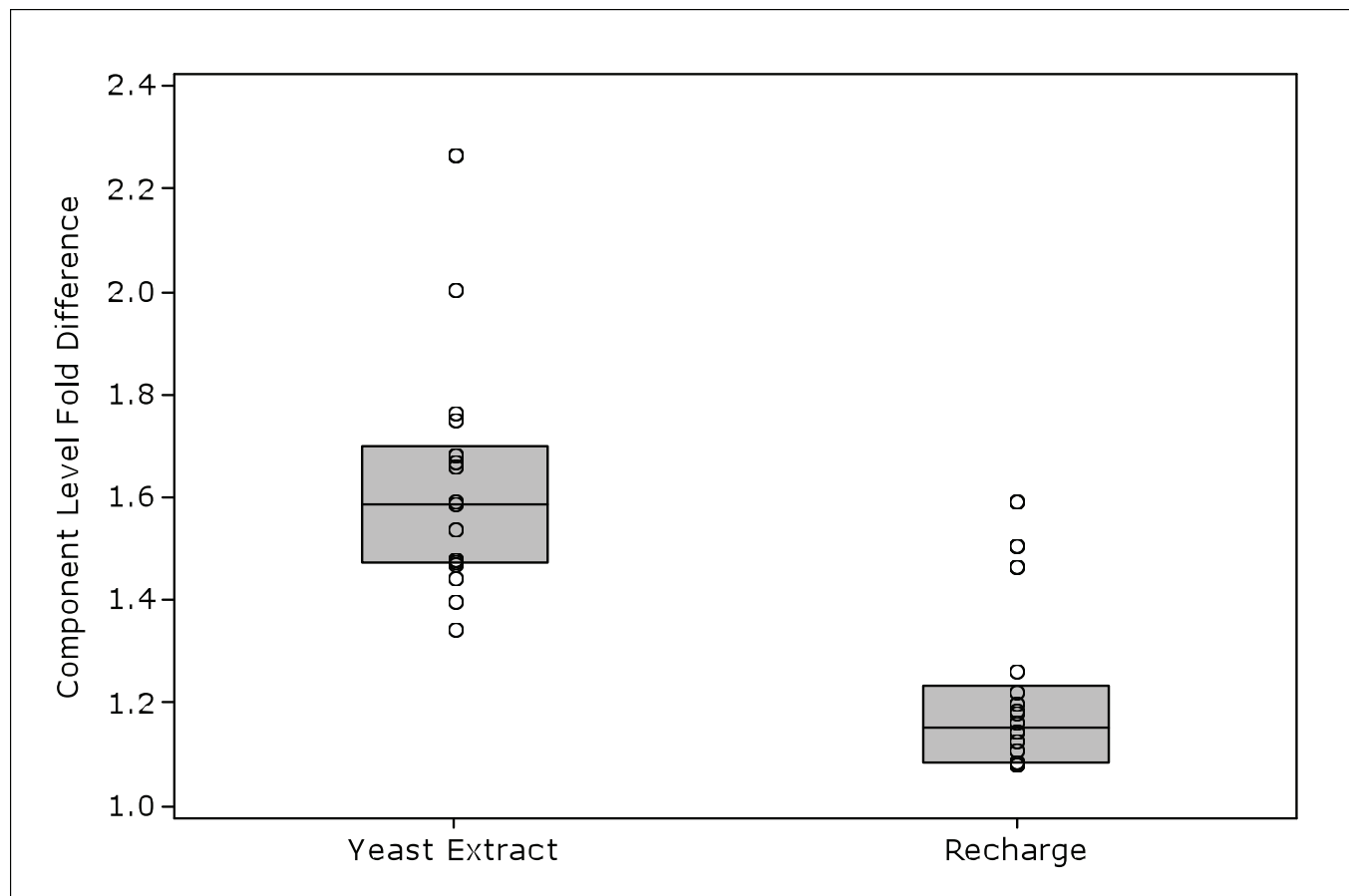


*Lot-to-lot Consistency*

An advantage of chemically defined supplements is the improved uniformity of composition over materials of biological origin, such as peptones. To assess the gain in consistency afforded by BD Recharge over peptone, the levels of functional components of several classes were quantitated in multiple lots of both BD Recharge and yeast extract using LC/MS methods. Representative quantitation data are shown in Figure 8. Within all 11 of the yeast extract lots tested, a minimum and a maximum concentration of each component across several classes was identified. To determine the variation of across classes, the ratio of the maximum to the minimum concentration across all lots was

calculated for the components in each class, yielding a fold difference value. The figure shows the maximum fold difference for each class compared to the same values determined for the four lots of BD Recharge. The variation among the component classes was in all cases lower for BD Recharge compared to yeast extract, with the maximum difference across lots being significantly lower for the chemically defined supplement. The median difference value for the peptone indicates that the component variability among the lots of yeast extract was clustered nearer to the minimum difference instead of being evenly distributed between the maximum and minimum.

**Figure 8:** Fold differences in component concentrations across multiple lots of yeast extract and BD Recharge. The largest ratio of maximum to minimum concentration within each component class, across all lots, is indicated by circles. The box indicates the middle 50th percentile of the fold difference data, with the median value shown by the horizontal line drawn through the box. If no variation is seen in component concentration, the fold difference would be 1.0. n = 11 lots of Yeast Extract, n = 4 lots of BD Recharge.



## DISCUSSION

### Formulation

The keys to making a successful cell culture supplement based on peptones were in identifying functional components in the hydrolysate, and in utilizing DOE mixture designs to find the optimum formulation to elicit a peptone-like response. The correct mixture of nutritional components and bioactive promoters of protein production, together with the elimination of potential performance inhibitors in the hydrolysate, combined to achieve this goal. Out of this strategy, a chemically defined, animal-free, and protein-free supplement was developed that provides growth and productivity performance equivalent to or better than peptones. This product has the benefit of high lot-to-lot consistency possible only with a fully chemically-defined formulation; the lack of animal-origin components further reduces risk. The safety margin with BD Recharge is also increased by the requirement for its components to be animal-free to the tertiary level so that it can be produced in BD's fully dedicated animal-free and antibiotic-free facility (AF<sup>2™</sup>).

### Performance

An important design consideration for BD Recharge was in generating a formulation that was generally applicable to different CHO expression systems, and not specific to only one platform.

BD Recharge shows good growth and production response in several CHO systems, including derivatives of CHO-K1, GS-CHO, or CHO DG44. The cell systems used in the design of BD Recharge produced a number of different recombinant proteins including mAbs, enzymes, and non-enzymatic proteins. In addition, the supplement shows performance benefits when used with many base media formulations, broadening its applicability across a wider range of culture conditions. As a general rule, BD Recharge can be initially tested at a concentration around half of that used with yeast extract; a pilot titration would be needed to determine the cell line optimum. When optimized, BD Recharge has been seen to increase protein production to levels that meet or exceed that seen with peptone, sometimes without the need to reach the same cell density (*i.e.*, with an increase in specific productivity).

Feeding approaches can also be used with BD Recharge to optimize the production increases with supplementation. Although the optimum fed-batch strategy needs to be determined for each cell line, it was found that the highest increases in productivity and viability occur when BD Recharge is included in the growth medium at day zero of the culture.

### *Protein Product Quality*

Protein quality—the collection of post-translational modifications that occur in a protein pharmaceutical during and after production—is a growing area of emphasis in the production and regulation of biotherapeutics. Because of this, it is important to assess the protein quality effects of a cell culture supplement used in the production of protein biologics. The reference for comparison of the BD Recharge performance, in this respect, is the protein quality seen with peptone supplementation. BD Recharge gives comparable results to peptones, as measured by charge-variant analysis, which suggests that it should yield similar product quality attributes if substituted for peptones in a process development project.

### *Summary*

It has been demonstrated that a chemically defined cell culture supplement can be developed by analysis and testing of the functional components of a peptone. The resulting supplement, BD Recharge, is animal-free, protein-free, and chemically defined, and boosts the productivity of a broad range of CHO host cell lines used in biopharmaceutical production. Although yeast extract was primarily used in these studies, other hydrolysates, such as soy or animal-origin peptones, could also be used in this process to yield additional chemically defined supplements with unique characteristics.

---

## **ACKNOWLEDGEMENTS**

AccQTag is a trademark of and ACQUITY UPLC and Alliance are registered trademarks of Waters Corporation. ©2011

Envision is a registered trademark of PerkinElmer Inc. ©2011 All Rights Reserved.

HiTrap is a trademark of GE Healthcare. ©2011

Octet is a registered trademark of ForteBio, Inc. ©2011 All rights reserved.

ProPac WCX-10 is a registered trademark of Dionex Corporation. ©2011 All rights reserved.

TPP TubeSpin is a registered trademark of TPP Techno Plastic Products AG. ©2011

Vi-Cell is a registered trademark of Beckman Coulter, Inc. ©2011

Recharge and AF<sup>2</sup> are trademarks of Becton, Dickinson and Company. ©2011 BD