ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Utilization of Tyrosine- and Histidine-Containing Dipeptides to Enhance Productivity and Culture Viability

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Received 17 November 2011; revision received 21 February 2012; accepted 12 March 2012

Published online in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bit.24507

ABSTRACT: Adequate supply of nutrients, especially providing a sufficient level of specific amino acids, is essential for cell survival and production. Complex raw materials such as soy hydrolysates or yeast extracts are the source for both free amino acids and peptides. However, typical chemically defined (CD) media provide amino acids only in free form. While most amino acids are highly soluble in media and can be provided at fairly high concentrations, certain amino acids such as tyrosine have poor solubility and thus, only a limited amount can be added as a media component. The limited solubility of amino acids in media can raise the risk of media precipitation and instability, and could contribute to suboptimal culture performance due to insufficient nutrient levels to meet cellular demands. In this study, we examine the use of chemically synthesized dipeptides as an alternative method for delivering amino acids to various monoclonal antibody producing cell lines. In particular, we focus on tyrosine-containing dipeptides. Due to their substantially higher solubility (up to 250-fold as compared with free tyrosine), tyrosine-containing dipeptides can efficiently provide large amounts of tyrosine to cultured cells. When tested in fed-batch processes, these supplemental dipeptides exerted positive effects, including enhanced culture viability and titer. Moreover, dipeptide-supplemented cultures displayed improved metabolic profiles including lower lactate and NH_4^+ production, and better pH maintenance. In bioreactor studies using two-sided pH control, a lactate spike occurring on day 10 and the concomitant high levels of base addition could be prevented with dipeptide supplementation. These beneficial effects could be obtained by one-time addition of dipeptides during inoculation, and did not require further feeds during the entire 11–15-day process. Non-tyrosine-containing dipeptides, such as His–Gly, also showed improved productivity and viability over control cultures.

Biotechnol. Bioeng. 2012;xxx: xxx–xxx.

KEYWORDS: dipeptides; amino acids; tyrosine; lactate; culture media, CHO cells

Introduction

Recombinant therapeutic proteins produced in mammalian cells represent a major class of biopharmaceuticals (Walsh, 2006). In recent years, therapeutic dosage demands have increased dramatically and are now driving the development of a variety of improvements to maximize expression levels for improved cost of goods. Over the years, advances in media and process optimization have resulted in significant improvements in expression levels. However, further optimization of nutritional supplements and medium additives are required to improve metabolism, cell survival and productivity, and ultimately, protein expression levels (Wurm, 2004). Chinese hamster ovary (CHO) cells are the most commonly used mammalian cells for production of proteins and antibodies (Birch and Racher, 2006). Historically, cell culture media for CHO cells relied on fetal bovine serum to meet growth and productivity requirements. However, use of serum in cell culture media poses a significant regulatory concern due to the potential contamination of animal-derived materials (ADMs) with viruses and prions. This risk stimulated development of ADM-free media, which in turn led to the use of protein hydrolysates as beneficial medium supplements (Kim and Lee, 2009). Protein hydrolysates or peptones are complex, undefined raw materials from plant or other non-animal sources. While lacking the risks of ADMs, their complex nature introduced a challenge in obtaining lot-to-lot consistency during manufacturing (Chun et al., 2007; Jan et al., 1994). Therefore, current efforts in medium

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Contract grant sponsor: Amgen, Inc.

development are focused on the use of chemically defined (CD) media for the production of recombinant proteins and monoclonal antibodies using CHO cells.

While protein hydrolysates pose a manufacturing challenge, they provide an optimal nutrient supply and are a source of free amino acids and bioactive peptides that exert specific effects on growth and productivity. Much effort has been devoted to identifying the individual components in peptones and their optimal concentrations (Chun et al., 2007; Franek et al., 2003; Jan et al., 1994). Franek et al. (2003) prepared fractions from plant protein hydrolysates and tested them for their ability to support growth and productivity in hybridoma cells. They further tested the activities of available synthetic peptides and reported beneficial effects of oligopeptides containing three to six amino acid residues on productivity and culture viability (Franek and Fussenegger, 2005; Franek and Katinger, 2002; Franek et al., 2003).

In this study, we evaluated several chemically synthesized dipeptides, focusing mostly on tyrosine-containing dipeptides. The reason for selecting tyrosine-containing dipeptides for evaluation was to investigate and possibly take advantage of the higher solubility of dipeptide forms, as compared to the low solubility of free tyrosine (Furst, 1998; Furst and Kuhn, 2000; Vazquez et al., 1993). Increasing the tyrosine content in media can prove challenging due to its low solubility. Even in the form of hydrated salt, tyrosine is only slightly soluble at neutral pH.

In contrast with the poor solubility of free tyrosine, dipeptides with a tyrosine constituent demonstrate significantly enhanced solubility. A tyrosine–histidine (Tyr–His) dipeptide has a reported solubility index of 100 g/L, which is an approximately 250-fold increase over free tyrosine with a reported solubility of only 0.4 g/L (Furst, 1998; Furst and Kuhn, 2000; Vazquez et al., 1993). In fact, tyrosine-containing dipeptides, as well as glutamine-containing dipeptides, are already being used in clinical nutrition to aid patients with renal or liver disease, or as formula supplements for infants (Druml et al., 1995; Furst, 1998, 2001; Furst and Kuhn, 2000; Vazquez et al., 1993). Clinicians are taking advantage of these supplemental dipeptides to deliver certain amino acids that are relatively unstable (such as glutamine) or poorly soluble (such as tyrosine) to benefit the patients. In a cell culture context, glutamine-based dipeptides have been tested and are commercially available as media supplements (Christie and Butler, 1994; Minamoto et al., 1991; Roth et al., 1988). In contrast, our literature search found only one study describing the effects of tyrosine-based dipeptides in cell culture (Erdmann et al., 2006). The authors indicate that methionine–tyrosine (Met–Tyr) can stimulate expression of the antioxidant defense proteins HO-1 and ferritin in endothelial cells and sustain antioxidant cellular protection.

The existing literature describing the high solubility (Furst, 1998; Furst and Kuhn, 2000; Vazquez et al., 1993) and potential antioxidant attributes of tyrosine-containing dipeptides led us to consider their use in cell culture processes. In addition, we also evaluated histidine-containing dipeptides, such as Gly–His and His–Gly, since literature reports that selective oligopeptides containing histdine possess pH-buffering and antioxidant capacities (Abe, 2000; Guiotto et al., 2005). We also evaluated the effect of a non-tyrosine, non-histidine containing dipeptide, threonine-phenylalanine (Thr–Phe), and compared its activity against various tyrosine and histidine containing dipeptides.

We report here for the first time the identification of synthetic dipetides that exert positive effects on multiple monoclonal antibody producing cell lines during fed-batch processes. In the present work, we supplemented CD media formulations with synthetic dipeptides that can be solubilized at relatively high concentrations. Our objective was to improve the culture nutrient supply and/or enhance the antioxidant and pH-buffering capabilities of the fed-batch media. We describe how dipeptide-supplemented cultures exhibited minimal nutrient depletion, improved metabolic profiles and pH maintenance, and enhanced viability and specific productivity. In particular, Tyr–His and Tyr–Lys displayed the most beneficial effects. Due to their high solubility at neutral pH, these dipeptides required only a single feed at the time of inoculation to provide a sufficient amount of tyrosine for the entire fed-batch period, in contrast with other tyrosine supplementation strategies that require multiple, separate tyrosine feeds. In fact, our dipeptide-supplemented cultures exhibited increased viability and specific productivity over control conditions beyond the process duration of 11 days, allowing extension of the culture up to 15 days using the same CD media. We also report beneficial effects of a non-tyrosine dipeptide, His–Gly, on culture performance.

Materials and Methods

Dipeptides

Chemically synthesized dipeptides were purchased from Bachem (Bubendorf, Switzerland). Each dipeptide was dissolved in room-temperature water (Nuclease-free water, Cat. No. AM9937; Ambion, Inc., Austin, TX) for preparation of a concentrated stock solution (10–100 mg/mL), followed by sterile filtration using 0.22 μm cellulose acetate filters (Cat. No. 430320; Corning, Inc., Corning, NY) or Spin-X filters (Cat. No. 8160; Corning, Inc.). On Day 0, prior to inoculation of production cell lines, appropriate volumes of concentrated dipeptide stock solutions were added to the production batch medium to bring the final dipeptide concentration to 0.125–2.0 g/L. For controls, the same volume of water was added to batch medium.

Shake Flask Experiments

A series of shake flask experiments were performed. Five different production cell lines, each expressing different

recombinant monoclonal antibodies, were tested with YH, YK, HG, and GH dipeptides. Culture performance of Cell Line B was further evaluated with tyrosine–alanine (YA), tyrosine–valine (YV) and threonine–phenylalanine (TF). In all cases, cells were inoculated at the same seeding density and cultured in the same incubator with the same condition. Bolus feeds of proprietary feed media were delivered on selective days. Cultures were harvested on Day 11. However, if viability on day 11 was $\geq$70%, the culture duration was extended by introducing a fourth feed. A fifth feed on day 13 was introduced if viability was $\geq$50%, with appropriate feed amount. Glucose was fed as needed with the target concentration used in a typical process. After Day 11, the glucose target was lowered appropriately to accommodate lower cell viability.

Bioreactor Experiment

A bioreactor experiment examining the performance of Cell Line B with tyrosine–histidine (YH) and tyrosine–lysine (YK) dipeptides was performed. Six 2-L bioreactors were run under similar conditions. A control condition was performed in duplicate, while the remaining reactors were fed 0.5 g/L YH, 1.0 g/L YH, 0.5 g/L YK, and 1.0 g/L YK (singlet for each condition). A typical production process was utilized. Inoculation density, temperature, and duration matched the shaker flask process. Two-sided pH control and a proprietary feed schedule were employed.

Media

For both shaker and bioreactor experiments, proprietary chemically defined production batch medium and feed medium were used. Prior to production, cells were cultured in passaging media plus appropriate methotrexate (MTX) concentrations. Approximately 20% carry-over from N-1 stage was inoculated to production batch media.

Assays

On selective days during the production run, small volumes of culture were taken to assess viable cell density and cell viability using the Cedex AS20 cell counter (Roche Innovatis, Beilefed, Germany). For the bioreactor experiment, samples were taken daily. Metabolic data were obtained from the Nova Bioprofile 100 Plus (Nova Biomedical, Waltham, MA), and an Advanced Instruments (Norwood, MA) osmometer model 2020 was used to measure osmolality. A Chiron Model 248 blood gas analyzer (Siemens Healthcare Diagnostics, Deerfield, IL) was used to measure pH, dissolved carbon dioxide, and dissolved oxygen.

Titer and amino acid content were also determined using conditioned media taken during the production duration. Centrifuged supernatant was frozen at -20°C before delivery to the Amgen Thousand Oaks (ATO) High Throughput Laboratory for titer and amino acid analyses. Titer values were measured using affinity Protein A HPLC. Appropriate extinction coefficient values were applied for each molecule to determine the final titer. The amino acid analysis method utilized the AccuTag reagent kit and pre-column derivatization chemistry. Derivatization, chromatography, and data analysis steps were performed according to vendor recommendations (Waters Corporation, Milford, MA).

Results

Increased Productivity and/or Culture Viability Is Observed With Tyr–His or Tyr–Lys Dipeptide Addition Across Multiple Cell Lines

When tyrosine–histidine (YH) or tyrosine–lysine (YK) dipeptides were added to the batch production media at a final concentration of 0.5–2.0 g/L, a significant improvement in titer (up to 37% increase) and culture viability (up to 67% increase) was observed in Cell Lines A and B in shaker flasks (Fig. 1). In control conditions (no dipeptides added), viability of Cell Line A dropped below 50% by Day 13. In contrast, viability was maintained above 50% in all dipeptide-containing cultures (Fig. 1B). The most effective result in terms of viability improvement was obtained when Tyr–His was added at 2 g/L final concentration, which resulted in 74% viability on day 13 (in contrast with 45% in the control culture). However, the highest titer was achieved when Tyr–His was supplemented at a lower concentration (1 g/L), resulting in a final day 14 titer 35% higher than the control condition (Fig. 1A). The lower titer obtained with higher Tyr–His concentration (18% higher than controls) is due to significant growth suppression (data not shown).

The beneficial effects of these dipeptides on culture performance were also observed in Cell Line B (Fig. 1C and D). A significant improvement in titer (up to 45% increase) and culture viability (up to 56% increase) was detected when either Tyr–His or Tyr–Lys was added to the production batch media at the time of culture inoculation. These results were consistent with those observed in Cell Line A. We also observed enhanced productivity and viability in other cell lines tested in shake flasks, including Cell Lines C, D, and E. While the extent of titer improvement in Cell Line C was relatively modest (up to 25% increase) in comparison with that observed in Cell Line A (up to 37% increase) or Cell Line B (up to 45% increase), the effect on culture viability was more dramatic (Fig. 1E and F). Dipeptide additives were able to prevent premature cell death occurring on day 10 in shakers and allowed the culture to be extended longer. Similar to Cell Line C, the extent of titer improvement was also modest in Cell Lines D and E, but a significant improvement in viability was observed (data not shown).

To determine whether the beneficial effect of Tyr–Lys and Tyr–His could be achieved by dipeptides in general, other

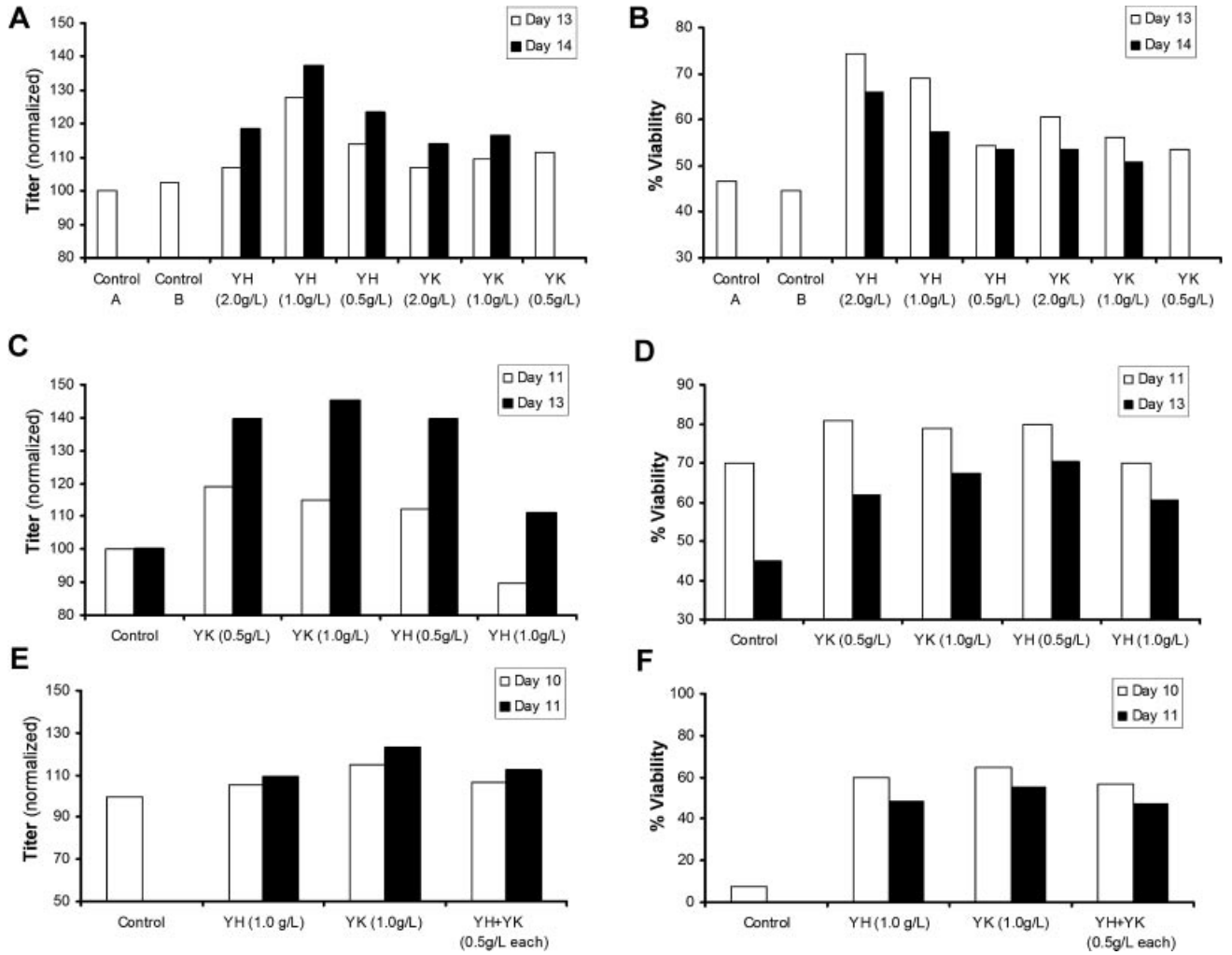

Figure 1. Increased productivity and/or culture viability is observed when different cell lines are supplemented with Tyr–His (YH) or Tyr–Lys (YK) dipeptides, with the indicated amounts added to the production batch media at the time of inoculation in shaker fed-batch experiment. All values represent normalized, relative values with arbitrary units. **A**: Titer (Cell Line A). **B**: Culture viability (Cell Line A). **C**: Titer (Cell Line B). **D**: Culture viability (Cell Line B). **E**: Titer (Cell Line C). **F**: Culture viability (Cell Line C).

synthetic dipeptides (including Thr–Phe, His–Gly, Gly–His, Tyr–Ala, and Tyr–Val) were screened in shake flask cultures, using Cell Line A. As shown in Figure 2, not all dipeptides were able to induce titer improvement. In fact, Gly–His had a dose-dependent negative effect on productivity. In contrast, a slight improvement in Cell Line B titer was observed with His–Gly addition, suggesting that the specific structure or orientation of a dipeptide's constituent amino acids can dictate its functional role. Interestingly, other tyrosine-containing dipeptides such as Tyr–Ala and Tyr–Val were also able to induce titer improvement, even at a lower concentration (Fig. 2). However, Tyr–Ala and Tyr–Val dipeptides demonstrated lower solubility in water as compared to Tyr–His or Tyr–Lys (data not shown). Therefore, Tyr–His and Tyr–Lys were chosen for evaluation at larger scale in 2-L bioreactors.

The beneficial effects of these synthetic dipeptides observed in shaker experiments (Fig. 1) were later reproduced in bioreactors (Fig. 3). In 2-L vessels, both Tyr–His and Tyr–Lys dipeptides extended culture duration (Fig. 3B) by improving culture viability, and significantly enhanced titer and specific productivity (Qp) in the pH-controlled bioreactor environment (Fig. 3A and C). Volumetric productivity (VPR) continued to increase beyond day 11 for dipeptide-supplemented cultures (Fig. 3D).

Improved Metabolic Profiles Are Observed in Dipeptide Supplemented Cultures

In correlation with titer and viability improvement, enhanced metabolic profiles were achieved with dipeptide addition. In the bioreactor experiment, the control reactors began accumulating lactate on day 10 (Fig. 4A), while dipeptide-supplemented bioreactors consumed lactate

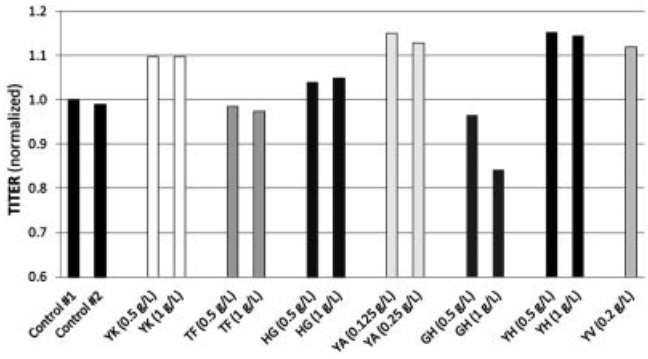

Figure 2. Only specific dipeptides are able to induce titer improvement in Cell Line B. Improvement in day 11 titer is observed with Tyr–Lys (YK), Tyr–Ala (YA), Tyr–Val (YV), and His–Gly (HG) addition, while a decrease in titer is detected with Gly–His (GH) addition in a dose-dependent manner. Thr–Phe (TF) addition had no significant effect on titer (shaker experiment). Normalized titer values are represented in arbitrary units.

during the same timeframe. Concomitant with lactate accumulation, the Na^+ levels also started to rise in the control reactors (Fig. 4B), reflecting exogenously added base in response to the drop in pH. However, the Na^+ level was well maintained in dipeptide-supplemented conditions. These data suggest that supplemental dipeptides can prevent media acidification occurring in the latter phase of production runs, thereby requiring less base addition.

Similar to bioreactor results for Cell Line B, improved lactate profiles were observed in shaker conditions (Fig. 4C and D). However, lactate consumption was observed only with a higher concentration (1.0 g/L YH) of dipeptide. When a lower amount (0.5 g/L YH) was added, lactate accumulation could not be prevented, but was delayed (Fig. 4C). These results correlate with the pH profile of the culture (Fig. 4D). At the higher dipeptide concentration (1.0 g/L), pH was maintained at a relatively

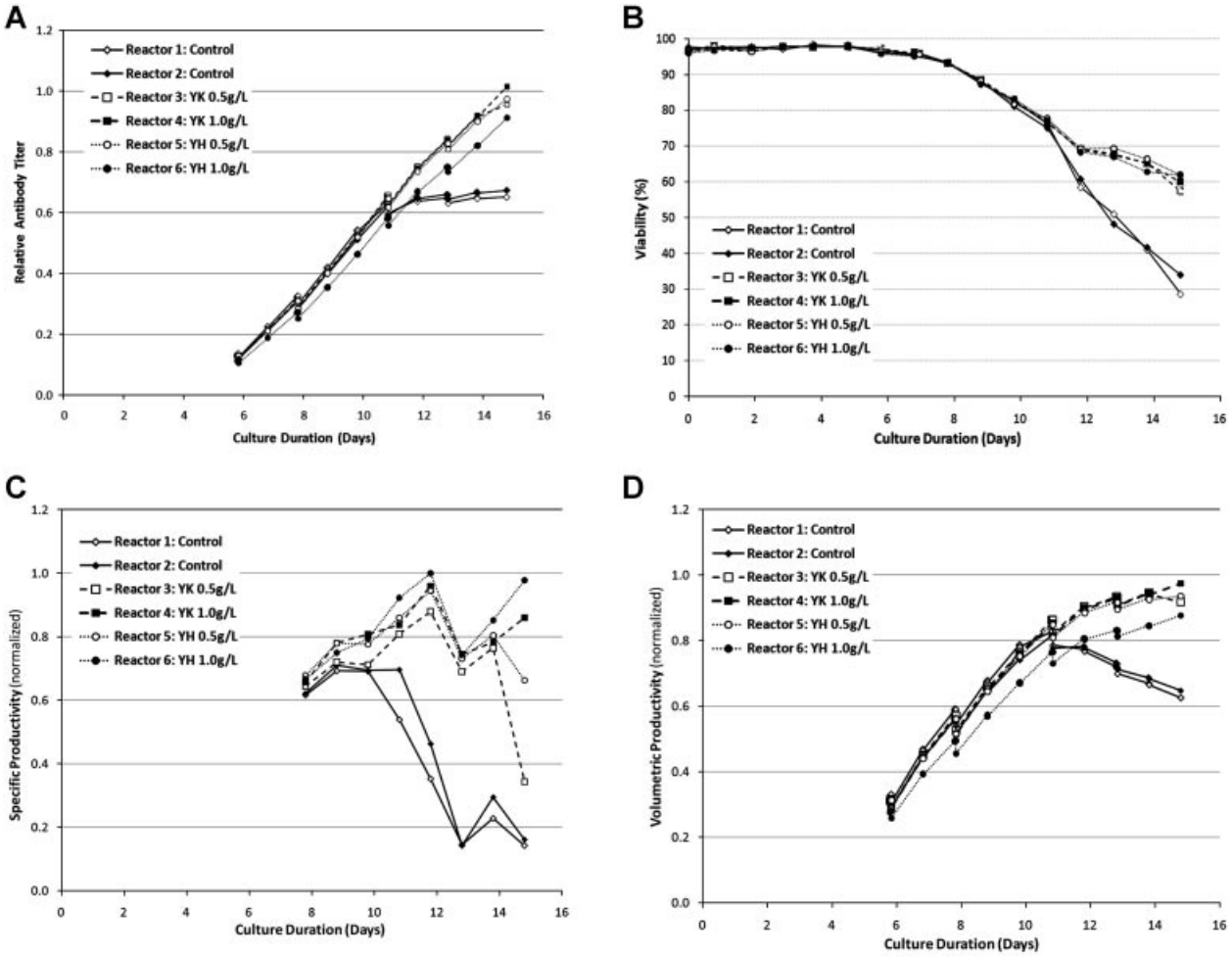

Figure 3. A significant improvement in productivity and culture viability is observed when Cell Line B is tested with Tyr–His (YH) or Tyr–Lys (YK) dipeptides with the indicated amounts of specific dipeptides added to production batch media at the time of inoculation in 2-L bioreactors (with two-sided pH control). **A**: Titer; **B**: %viability; **C**: specific productivity; **D**: volumetric productivity. Normalized, relative values are represented with arbitrary units.

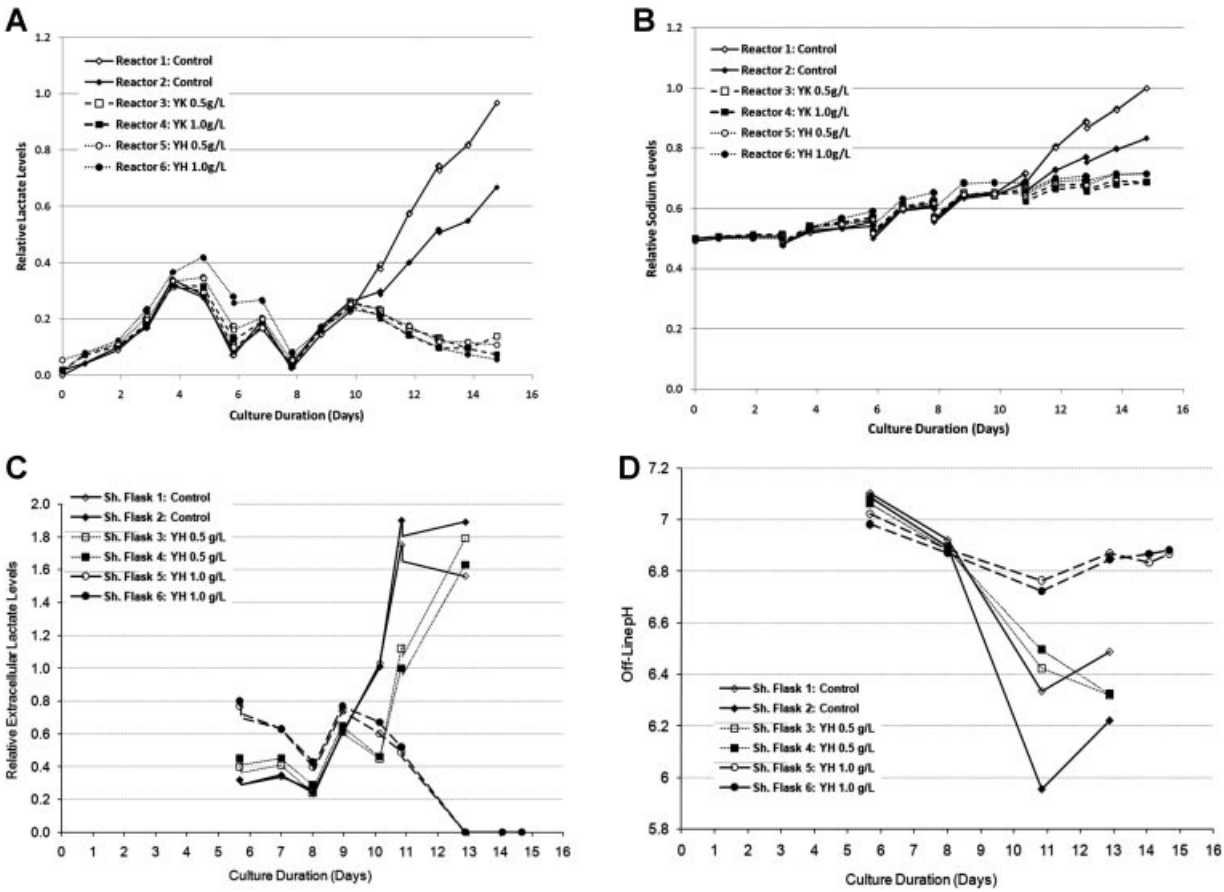

Figure 4. Improved metabolic profiles of Cell Line B in 2-L bioreactors and shakers with YH or YK dipeptide addition. **A**: Lactate profile in bioreactors; **B**: sodium (Na^+) profile in bioreactors; **C**: lactate profile in shakers; **D**: pH profile in shakers. Normalized, relative values are represented with arbitrary units.

constant level while at the lower dipeptide concentration (0.5 g/L), a significant decrease in pH was detected. However, the extent of this pH drop was less severe in the lower dipeptide concentration condition than in the "control" condition (Fig. 4D). Since shaker conditions do not have active pH control, higher levels of dipeptide may be required to achieve a similar level of benefit as in the bioreactors.

Improved NH_4^+ profiles were also observed in dipeptide-supplemented conditions (data not shown). However, similar to the lactate profiles, shaker conditions were more sensitive to dipeptide concentrations than bioreactors, in terms of reducing NH_4^+ levels.

Tyrosine Depletion Is Prevented in Cultures Supplemented With Tyr-Containing Dipeptides

Amino acid analyses from the bioreactor experiment using Cell Line B indicated that in control reactors, both tyrosine (Tyr) and asparagine (Asn) are depleted by day 11 (Fig. 5A and B). In contrast, tyrosine depletion was not detected in Tyr–Lys or Tyr–His supplemented conditions (Fig. 5A). However, neither of these synthetic dipeptides could rescue the cultures from asparagine depletion (Fig. 5B). In contrast to the tyrosine profile, which differed significantly between the control and dipeptide conditions (Fig. 5A), the asparagine profile remained relatively comparable among different conditions (Fig. 5B). The eventual depletion of tyrosine on day 15 was observed with lower dipeptide levels (0.5 g/L of either YH or YK), while cultures with higher dipeptide concentrations (1.0 g/L of either YH or YK) never experienced tyrosine depletion during the 15-day culture period (Fig. 5A). These results suggest that free tyrosine is being liberated from dipeptides during the course of production. With higher dipeptide supplementation (1.0 g/L), a higher amount of free tyrosine became available (Fig. 5A). Since neither Tyr–Lys nor Tyr–His contains the asparagine moiety, asparagine levels were not affected (Fig. 5B). Interestingly, higher levels of free tyrosine was observed in 1.0 g/L YH condition than the 1.0 g/L YK condition. This may be partially due to repressed

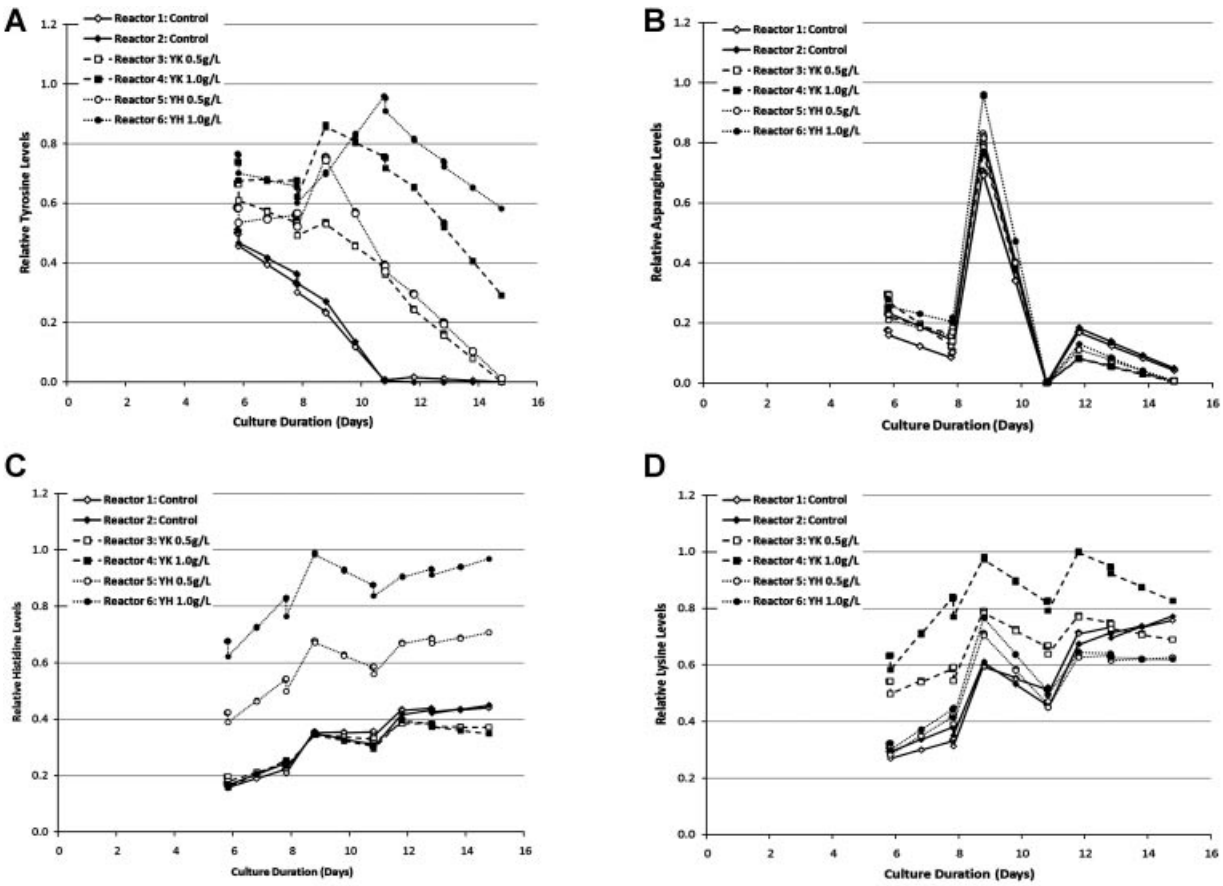

Figure 5. Amino acid profiles of Cell Line B in 2-L bioreactors with and without YH or YK dipeptide addition. Normalized, relative values are represented with arbitrary units. **A**: Tyrosine (Tyr) profile comparison. Tyrosine levels are depleted by day 11 in control bioreactors, but not with dipeptide addition. **B**: Asparagine (Asn) profile comparison. Asparagine depletion was detected in all conditions of 2-L bioreactors. YK and YH dipeptides could not prevent Asn depletion. **C**: histidine (His) profile comparison in 2-L bioreactors. A substantial increase in His levels was detected only in YH-supplemented conditions, in a dose-dependent manner. **D**: Lysine (Lys) profile comparison in 2-L bioreactors. A substantial increase in Lys levels was detected in YK-supplemented conditions.

cell growth observed in the 1.0 g/L YH condition (data not shown).

Higher histidine (His) levels were observed in His-containing dipeptide conditions only (i.e., Tyr–His), further supporting the idea that these dipeptides are being dissociated over time into free amino acids (Fig. 5C). A very distinct difference in the histidine levels was observed between lower (0.5 g/L) versus higher (1.0 g/L) YH conditions, even as early as day 6 (Fig. 5C). Lysine profiles also displayed a dose-dependent increase in free lysine levels in YK-supplemented conditions (Fig. 5D). However, the difference in lysine levels was less obvious in the 0.5 g/L YK-fed condition as compared to conditions receiving no YK supplement. For example, the free lysine levels detected in YH-supplemented conditions (either at YH 0.5 g/L or YH 1.0 g/L) is similar to the lysine level detected in the 0.5 g/L of YK-supplemented condition on day 9 and also on day 15 (Fig. 5D). This could be due to inefficient consumption of lysine by YH-treated cultures.

Tyrosine Availability Is Correlated With Specific Productivity

Bioreactor results suggest that tyrosine depletion in Cell Line B has a deleterious effect on specific productivity (Qp). Tyrosine depletion observed on day 11 (Fig. 5A) in the control reactors correlates with a sharp decrease in Qp (Fig. 3C). In contrast, an increase in Qp is observed in dipeptide-supplemented conditions with higher levels of tyrosine available. This functional correlation between tyrosine and Qp is again observed between days 14 and 15, during which a sharp decline in Qp is observed in cultures supplemented with lower dipeptide concentrations (i.e., in YK 0.5 g/L and YH 0.5 g/L supplemented conditions; see Fig. 3C). Amino acid profiles indicate that during this timeframe, tyrosine is completely depleted in 0.5 g/L dipeptide conditions (Fig. 5A). In contrast, sufficient levels of tyrosine were available in 1.0 g/L dipeptide conditions on Day 15, and high Qp was maintained (Fig. 3C).

Discussion

In this study, we evaluated several dipeptides as supplements to our chemically defined (CD) media. While tyrosine is difficult to dissolve in the media, both Tyr–His and Tyr–Lys dipeptides demonstrated high solubility, and we were able to prepare concentrated stock solutions (100 g/L) in order to minimize the volume added to the culture at inoculation. No separate or additional feed of dipeptide was necessary after the initial supplement. Amino acid analyses indicate that the amount added to the batch media for shakers and bioreactors at inoculation was sufficient to provide enough tyrosine for the entire culture duration of 13–15 days (Fig. 5A). The high solubility of Tyr–His or Tyr–Lys at neutral pH allows for their direct integration into the basal CD medium, and has the potential to be incorporated into the feed medium if a higher tyrosine concentration is desired. This solubility benefit can ultimately translate into the development of manufacturing-friendly and operationally efficient process, especially in a large-scale production setting.

In addition to their solubility benefits, these tyrosine-containing dipeptides improved culture productivity and viability across multiple cell lines (Fig. 1), and enhanced metabolic phenotypes in both shaker and bioreactor settings (Fig. 4). Tyrosine dipeptides could efficiently delay or prevent lactate accumulation (Fig. 4A and C) and allowed for better pH maintenance (Fig. 4B and D) in both shaker and bioreactor contexts. It is yet to be determined whether tyrosine-containing dipeptides exert positive effects solely by providing more tyrosine or whether they also confer additional benefits such as antioxidant activity or pH-buffering capacity. However, the observation that selective dipeptides can also enhance the viability and productivity of cell lines that do not experience tyrosine starvation suggests they exert positive effects beyond maintenance of tyrosine levels. This is consistent with literature that reports antioxidant effects of the dipeptide Met–Tyr in cell culture (Erdmann et al., 2006). While we have not directly compared the effect of providing free amino acids against the beneficial effect of dipeptides, this same article reports that the addition of single amino acids (i.e., methionine or tyrosine) to cultures did not exert the same antioxidant effects as the Met–Tyr dipeptide (Erdmann et al., 2006).

Non-tyrosine containing dipeptides displayed a range of effects on cultures. His–Gly was able to improve productivity and culture performance in Cell Lines A (data not shown) and B (Fig. 2). However, Gly–His had a negative effect, suggesting that specific structural features of the dipeptide may be an important factor. These results also indicate that not all histidine-containing dipeptides are beneficial, and certain dipeptides can actually confer negative effects. Considering there was no histidine or glycine depletion in the control conditions, the positive effect of His–Gly dipeptide is likely to be independent of the glycine or histidine amino acid levels. Thr–Phe dipeptide, which contains neither tyrosine nor histidine, had no significant effect on the culture performance (Fig. 2).

Although we were not able to evaluate the kinetic characteristics of these dipeptides, several studies report putative mechanisms for their catabolism. For example, literature reports the existence of aminopeptidases which catalyze the hydrolysis of dipeptides into their constituent amino acids (Christie and Butler, 1994; Vazquez et al., 1993; Werynski et al., 2006). Membrane-bound aminopeptidases can facilitate the hydrolysis of dipeptides outside the cell, or dipeptides can enter cells intact and then undergo hydrolysis by intracellular peptidases. It is yet to be determined what portion of the YH or YK dipeptides is cleaved extracellularly to provide the observed increase in tyrosine content. It is also unknown how quickly these dipeptides are being hydrolyzed or transported into the cells. A gradual rather than rapid hydrolysis of dipeptides in the medium has been proposed as a probable mechanism (Christie and Butler, 1994; Werynski et al., 2006), although kinetic studies in rats and humans demonstrate the rapid disappearance of dipeptides from plasma when injected intravenously (Vazquez et al., 1993). However, the observed fast clearance may reflect rapid transport of dipeptides into the cells rather than rapid hydrolysis. We have yet to evaluate how these dipeptides are being metabolized by different cell lines during fed-batch processes. The gradual release of tyrosine from the dipeptides, if indeed the case, could mimic a strategy in which tyrosine is fed in a continuous mode rather than as a bolus feed. This would reduce potential osmotic shock which the cells may experience from a bolus feed.

A functional correlation between tyrosine depletion and decreases in specific productivity (Qp) was observed in this study (Figs. 5A and 3C). A possible explanation for the observed deleterious effect of tyrosine depletion with respect to productivity lies in the metabolic fate of tyrosine. Tyrosine can be metabolized into fumarate and acetoacetate through a series of enzymatic reactions (Lehninger, 2005). Although fumarate can be generated from succinate via succinate dehyrogenase (SDH), having a secondary pathway for fumarate generation might be important, especially if SDH levels or activity become a limiting factor. SDH, also known as Complex II or succinate:quinone oxidoreductase, is a unique enzyme with dual roles, participating in both the tricarboxylic acid (TCA) cycle and the Electron Transport Chain (Oyedotun and Lemire, 2004). As such, it is prone to a wide range of inhibitors and oxidative damage. Providing a sufficient level of tyrosine may permit the cells to bypass a strict dependency on SDH as the only source for fumarate generation. In addition, tyrosine is a metabolic precursor for acetoacetate, which in turn can be converted to acetyl–CoA, an important molecule which can either be fed into the TCA cycle for energy production, or used for fatty acid synthesis. Thus, tyrosine may be seen as an efficient source for energy production for cells, and may serve to meet cellular energy requirements during monoclonal antibody production.

In conclusion, we have shown that selective dipeptides can provide multiple benefits, including enhanced productivity,

viability and improved metabolic profiles. Further possibilities to increase productivity include implementing higher seeding densities and development of improved versions of chemically defined media. With these changes, a higher demand for crucial nutrients such as tyrosine will have to be met. Chemically synthesized dipeptides could provide an efficient means to deliver these requirements, while possibly exerting additional benefits including antioxidant and/or improved buffering capacity for enhanced productivity and culture performance.

The authors wish to thank the ATO High Throughput Lab for their support for titer analysis (Dazy Johnson and Mee Ko) and amino acid analysis (Eugene Babcock and Janice Chen), the ATO Media Support Group for their assistance with various media preparations, and Todd Luman for providing valuable suggestions and input for bioreactor-related operations. Research funding was provided by Amgen, Inc.

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