Production of Butyrate and Branched Chain Amino Acid Catabolic Byproducts by CHO Cells in Fed-batch Culture Enhances their Specific Productivity

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Abstract

Chinese hamster ovary (CHO) cells in fed-batch cultures produce several metabolic byproducts derived from amino acid catabolism, some of which accumulate to growth inhibitory levels. Controlling the accumulation of these byproducts has been shown to significantly enhance cell proliferation. Interestingly, some of these byproducts have physiological roles that go beyond inhibition of cell proliferation. In this study, we show that, in CHO cell fed-batch cultures, branched chain amino acid (BCAA) catabolism contributes to the formation of butyrate, a novel byproduct that is also a well-established specific productivity enhancer. Further, the other byproducts of BCAA catabolism, isovalerate and isobutyrate, which accumulate in CHO cell fed-batch cultures also enhance specific productivity. Additionally, the rate of production of these BCAA catabolic byproducts was negatively correlated with glucose uptake and lactate production rates. Limiting glucose supply to suppress glucose uptake and lactate production, like in case of fed-batch cultures employing HiPDOG technology, significantly enhances BCAA catabolic byproduct accumulation resulting in higher specific productivities.

Keywords

CHO Cells; Branched chain amino acid; Short chain fatty acid; Specific productivity; growth inhibitors

Introduction

A variety of mammalian cells lines are currently being used for production of biologics, the market for which is around US $188 bn according to current estimates (Walsh, 2018). Of these cell lines, Chinese hamster ovary (CHO) cells are the leading candidates for production of the recombinant proteins used as

therapeutics or prophylactics (Walsh, 2018). Glycosylation patterns of proteins produced in CHO cells are similar to those produced in humans, which is one of the drivers for adoption of CHO cells as a protein production host cell line (J. Y. Kim, Kim, & Lee, 2012). In addition, CHO cells offer other advantages including plasticity to adapt to various selective pressures, anchorage independent growth in serum-free chemically-defined medium, resilience to harsher growth environments typically observed in production stages of cell culture processes (high lactate, high osmolality), reduced susceptibility to certain viral infections, etc (Dhara, Naik, Majewska, & Betenbaugh, 2018; Dumont, Euwart, Mei, Estes, & Kshirsagar, 2016). CHO cells also express a number of central metabolism enzymes that can allow them to make metabolic adjustments to maintain intracellular homeostasis in response to changing biochemical environment during cultivation (O'Brien, 2020). CHO cells can also produce mAbs at specific rates that rival the natural antibody producing cells of the human immune system, plasma cells. All the above properties, when combined with advancement and intensification of cell culture medium and process technologies that the bioprocessing field has seen in the last two decades, allowed the CHO based process productivities for most monoclonal antibodies to reach 5-10 g/L range and beyond.

While plasticity and resilience are positive attributes, the metabolic phenotype of CHO cells, which includes a predilection for the Warburg effect, presents several challenges in development of robust cell culture processes. CHO cells consume large amounts of nutrients and divert most of them towards byproduct production (Pereira, Kildegaard, & Andersen, 2018) such as lactic acid and ammonia. These two well-established metabolic waste products can accumulate to significant levels in fed-batch cultures and negatively impact cell growth and productivity (Cruz, Freitas, Alves, Moreira, & Carrondo, 2000; Lao & Toth, 1997; Yang & Butler, 2000). Knowledge of CHO cell physiology and cellular metabolism is critical for development of intensified cell culture processes which can be robustly scaled up to manufacturing scale. Utilizing an enhanced understanding of central metabolism of CHO cells, control strategies have been previously devised and successfully implemented to limit production of above-mentioned classical

byproducts. For example, HiPDOG technology was developed to control lactate production by using the near-exclusive dependence of cells on glucose availability to produce lactate (Gagnon et al., 2011). Similarly, the glutamine synthetase expression system is widely used to develop producing cell lines (Fan et al., 2012) that divert amino acid catabolism derived ammonia towards biosynthesis of much needed glutamine and away from release into the culture milieu.

More recently, Omics and systems biology techniques are being used to further elucidate mammalian cell physiology and metabolism (Hefzi et al., 2016; Mulukutla, Gramer, & Hu, 2012; Sumit et al., 2019). Employing metabolomics techniques, our group has previously identified several, formerly unknown, CHO cell metabolic byproducts that accumulate in fed-batch cultures (Mulukutla, Kale, Kalomeris, Jacobs, & Hiller, 2017). Some of these metabolic byproducts accumulate to concentrations that inhibit cell proliferation. These growth-inhibitory metabolites were determined to be either intermediates or byproducts of amino acid catabolism. By controlling the residual levels of specific amino acids in a low concentration range (0.5 – 1mM) during the growth phase of fed-batch cultures, we demonstrated that production of the corresponding byproducts could be significantly reduced. The result was an extension of the growth phase of the fed-batch cultures with a significant increase in peak cell densities and a corresponding increase in productivity. Ensuing work involved development of genetic engineering approaches to limit the production of these metabolic products (Mulukutla et al., 2019). The minimally expressed enzymes in the main catabolic pathway of phenylalanine-tyrosine (Phe-Tyr), as determined by transcriptomic analysis, were ectopically expressed in CHO cells. This resulted in reduced channeling of metabolic intermediates, specific this pathway, towards biosynthesis of growth inhibitory byproducts. In case of the BCAA catabolic pathway, the gene encoding the first enzyme in the BCAA catabolic pathway, namely branched chain amino acid transaminase 1 (BCAT1), was knocked-out from CHO cells eliminating production of three downstream inhibitory byproducts. These engineering attempts resulted

in significant extension of the growth phase and length of the culture and, yielded higher viabilities, peak cell densities and titers.

The physiological roles of these novel byproducts in context of CHO cell bioprocesses haven't been explored beyond growth inhibition. In addition, the dynamics of production of these metabolic products is still an outstanding question. The current study focuses on deciphering physiological roles of three metabolic byproducts of BCAA catabolism and the dynamics in their production/accumulation. Two of these byproducts are previously discovered, namely, isovalerate and isobutyrate (Mulukutla et al., 2019). The third metabolic product is butyrate, for which we show evidence that establishes its accumulation in CHO cell fed-batch cultures and supports BCAA catabolism a key metabolic source. Further, we show that in addition to butyrate, which is an established cell-specific productivity (q_p) enhancer, isovalerate and isobutyrate can also enhance q_p of CHO cells. We also show that the specific production rates of these three metabolites are negatively correlated to the rates of glucose consumption and lactate production. Finally, we show that use of glucose limitations strategies, like HiPDOG, can enhance production of these metabolites and, thereby, enhance q_p in the production stages of CHO cell fed-batch cultures, resulting in higher overall productivities.

Materials and Methods

Cells, Medium, and Fed-shake Cultures

Twelve CHO cell lines (Cell Line A, B, C, D, E, F, G, H, I, J, K and an ATCC CHO K1 derived cell line) utilizing a glutamine synthase expression system and each expressing a different recombinant antibody were used in the experiments described in this study. Two types of proprietary medium were used: "Medium A" is the production medium which is used for inoculation of the HiPDOG or conventional non-glucose-limiting fed-batch experiments on day 0 of the culture. The only differences between "Medium A" used

in a HiPDOG or traditional fed-batch culture were the starting glucose and sodium bicarbonate concentrations. “Medium B”, which is an enriched nutrient solution, was used as a feed medium for HiPDOG and fed-batch processes.

Medium A is a chemically defined, protein-free, amino acid fortified version of DMEM:F12 medium with adjusted levels of vitamins, trace elements, sodium bicarbonate and potassium chloride, and containing polyvinyl alcohol. Medium B is chemically defined, protein-free concentrated (feed) medium with amino acid, vitamin and trace elements (a selection of those in Medium A) at levels 3-3.5-fold higher on average, than the corresponding levels in A. For HiPDOG cultures using BCAT1 KO clones, Medium B was supplemented with additional amounts of tyrosine, cysteine, zinc and alanine in order to avoid any potential depletion of these compounds in the medium of these cell culture processes due to higher cell densities attained.

Purified compounds of isobutyric acid, isovaleric acid, and 2-methylbutyric acid were obtained from Sigma-Aldrich® for add-back experiments. Purified butyric acid sodium salt was obtained from Acros Organics (Fair Lawn, NJ).

Calculations for Specific Rates

Specific productivity (q_P) is the rate of protein produced per cell per unit time. It was calculated by taking product concentration in the culture supernatant at a given timepoint and dividing by the integrated viable cell concentration (IVCC) of the culture at that time point (units: 1E6 cells*day/mL).

Specific lactate production rate is the rate of lactate produced per cell per unit time. It was calculated by taking the concentration of lactate in the bioreactor at that timepoint and dividing by the integrated viable cell concentration (IVCC) of the culture at that time point (units: 1E6 cells*day/mL).

Specific short chain fatty acid (SCFA) production rate is the rate of total SCFA produced per cell per unit time. It was calculated by taking the sum of the SCFAs (butyrate, 2-methylbutyrate, isobutyrate and isovalerate) concentration at that time in culture, and dividing by the approximate integrated viable cell concentration (IVCC) of the culture at that time point (units: 1E6 cells*day/mL).

Bioreactor Setup

2 L glass bioreactors (Applikon, Inc. Schiedam, Netherlands) with 1.0 L working volumes were equipped with Hamilton EasyFerm Plus pH probes and VisFerm DO probes (Hamilton, Reno, NV) linked to Bionet controllers (Broadley James, Irvine, CA) which maintained pH and dissolved oxygen. In all the fed-batch bioreactor conditions, HiPDOG control strategy was employed unless stated otherwise (for details on HiPDOG technology, see (Gagnon et al., 2011)). For CHO cell lines A, B, E, F, G, H, I, J and K the pH dead-bands used during and after HiPDOG control was operational were 7.025 +/- 0.025 and 7.05+/-0.15, respectively. These pH deadbands were also used for the ATCC CHO K1 derived cell line. For CHO cell line D, the pH dead-bands used during and after HiPDOG control was operational were 7.175 +/- 0.025 and 7.2+/-0.15, respectively. In CHO cell line C, the HiPDOG control strategy was only operational for short period (~1 day), and the culture was switched to a traditional fed-batch where pH was maintained between 7.0 and 7.2. The production medium used was Medium A and the feed media used was Medium B. Feed medium was added through the HiPDOG control strategy when the HiPDOG control was operational (day 0 to day 5-7), and manually at a set rate after the end of HiPDOG control. The inoculum cell densities used were 1E6 cells/mL, 1.2E6 cells/mL, 2.5E6 cells/mL or 4E6 cells/mL (cell line C), the starting working volume used was 1L, the amount of feed medium added by the end of the culture ranged between 25-50% of the starting volume and, the temperature and power per unit volume (used for determining agitation rate) used were 36.5 ^{o}C and 80 watts/m^{3}, respectively. While the HiPDOG control was operational (day 0 to day 5-7), the initial available glucose was consumed by the

cells naturally until the glucose level fell to a point at which point the cells began to also consume lactic acid (observed by a slight rise in pH of the culture). This commenced the feeding using HiPDOG control feeding strategy. After HiPDOG control was ended, the glucose levels were maintained at concentrations above 1 g/L by feeding glucose semi-continuously as necessary. The method used to run cell line A in hybrid perfusion fed-batch process listed in **Table 1** is described in a previous publication (Hiller, Ovalle, Gagnon, Curran, & Wang, 2017).

Viable cell density, glucose, lactate, and ammonia concentrations in the cell culture medium were measured daily using a Nova Bioprofile FLEX Analyzer (Nova Biomedical, Waltham, USA). Titer analysis was performed by Protein A HPLC (model 1100 HPLC, Agilent Technologies, Inc., Santa Clara, CA, protein A column model 2-1001-00, Applied Biosystems, Foster City, CA). For the metabolite byproduct analysis, spent media samples were collected on different days along the culture and were analyzed by NMR, LC-MS and GC-MS techniques as described in a previous publication (Mulukutla et al., 2017). Amino acid analysis was performed on spent medium using a method described in a previous publication (Mulukutla et al., 2017).

For bioreactor conditions that were part of the experiments investigating the effect of amino acid restriction, Medium A used for the low amino acid conditions, Low 8AA or Low 10AA, was modified to contain eight or ten amino acids at 0.6 mM concentrations, respectively. In Low 8AA, the eight amino acids included Phe, Tyr, Met, Trp, Leu, Ser, Thr and Gly. In Low 10AA, the ten amino acids included Ile and Val in addition to the eight amino acids listed for Low 8AA. Original Medium A was used for the control conditions (normal levels of amino acids). For all HiPDOG conditions, the HiPDOG strategy was operational from the start of the culture until Day 5 through 7. Rather than delivering feed medium containing multiple nutrients using the HiPDOG control strategy, as employed by other experiments in this study, for conditions in this experiment, a glucose only stock solution (300g/L glucose in water) was

delivered using the HiPDOG control strategy when the HiPDOG control was operational. After HiPDOG control was ended, glucose was maintained above 1 g/L by delivering necessary amount of glucose stock solution in a semi-continuous manner. Feed medium was delivered at a user defined constant specific rate (feed delivered on a per cell basis) throughout the culture starting on Day 1. A modified version of Medium B with adjusted levels of the above mentioned eight or ten amino acids was used as the feed medium for both low amino acid and control conditions. The levels of these eight or ten amino acids were adjusted in Medium B based on the strategy reported previously (Mulukutla et al., 2017). This strategy uses pre-determined exponential growth phase specific amino acid consumption rates and the user determined constant specific feed rate to assess the feed medium (Medium B) concentrations for the eight or ten amino acids. Delivering the modified Medium B at the fixed specific feed rate to the low amino acid condition should maintain levels of these eight or ten amino acids between 0.5 – 1 mM during the exponential growth phase of the culture. For low amino acid and control conditions, the starting working volume used, and the settings for temperature, pH, and impeller agitation were same as those mentioned earlier in this section, besides the experiments that were performed in ambr® 15.

For **Figure S2**, culture was removed from a Low 8AA HiPDOG bioreactor on day 6 and were moved to shake flasks and cultivated till day 14. The shake flasks were treated on day 6 either with isovalerate, butyrate, neither (control) or both compounds added at concentrations described in results section. The shake flasks were agitated at 120 rpm in a humidified incubator (Thermo Fisher Scientific, Waltham, MA) maintained at 36.5℃ and 5% carbon dioxide levels and fed daily boluses of feed of Medium B to match the daily feed % added during a typical bioreactor process. For **Figure S3**, culture was removed from a Low 10AA HiPDOG bioreactor running a process as described above and placed into an ambr® 15 (~15 mL working volume micro bioreactor, Sartorious, Göttingen, Germany). The ambr® 15 was kept at 36.5℃ and agitated at 1200 rpm. It received multiple boluses of feed per day to match the daily feed % typically added during the bioreactor process.

For **Figure 1I**, cell line C was cultivated in a non-glucose-limiting fed-batch mode in an ambr® 15. Cells was seeded at 4E6 cells/mL and the ambr® 15 was kept at 36.5℃ and agitated at 1200 rpm. Basal mediums were different for each of the low amino acid conditions where the starting concentrations of the amino acids being limited were 0.6 mM. In the control condition, the branched chain amino acids had starting concentrations over 4 mM. The cultures received multiple boluses of feed per day, starting on day 2. The feeding strategy of this experiment was to replicate the cell specific feed rate of the Low 10AA 1L bioreactor experiment performed with cell line C.

Results

CHO Cells in Culture Produce Short Chain Fatty Acids (SCFAs) that are Known to be HDAC inhibitors

CHO cells have been previously shown to produce SCFAs, including isovalerate, isobutyrate and 2-methylbutyrate, in fed-batch cultures (Mulukutla et al., 2019). On analyzing the HiPDOG cultures (fed-batch culture employing HiPDOG strategy) of six CHO cell lines (cell line A through cell line E and an ATCC CHO K1 derived cell line), it was observed that the above mentioned SCFAs accumulated in the extracellular milieu over the course of the fed-batch culture (**Table 1**). Analysis of day 12 samples from cell line A cultivated in a hybrid perfusion fed-batch culture showed that the above-mentioned byproducts accumulated to higher concentrations when compared to fed-batch cultures **(Table 1)**. Isovalerate and isobutyrate have been previously reported to impact histone acetylation (McBain, Eastman, Nobel, & Mueller, 1997). This has been hypothesized to be due to inhibitory effect of these compounds on the activity of Class I and Class IIa histone deacetylases (HDAC) (H. J. Kim & Bae, 2011). Sample from HiPDOG cultures of above-mentioned cell lines were further analyzed using NMR approaches to identify other potential HDAC inhibitors that might be accumulating over the course of

the culture. Interestingly, it was observed that butyrate, a known HDAC inhibitor, also accumulated with time in these cultures. The nutrient source of isovalerate, isobutyrate and 2-methylbutyrate production has been previously shown to be leucine, isoleucine and valine, respectively (Mulukutla et al., 2017; Mulukutla et al., 2019). However, though butyrate could be a (by)product of glucose fueled fatty acid synthesis (as per basic biochemistry), it is not clear if other nutrients could be contributing to butyrate production in CHO cell fed-batch cultures.

Branched Chain Fatty Acid Catabolism Contributes to Butyrate Production

It was previously shown that production of isovalerate, isobutyrate and 2-methylbutyrate in HiPDOG cultures can be reduced by maintaining the residual levels of leucine, valine and isoleucine in a low concentration range (Mulukutla et al., 2017). As expected, controlling the concentrations of leucine, isoleucine and valine (**Figure S1**), in addition to few other amino acids, in low concentration range (0.5-1mM) in HiPDOG cultures of CHO cell lines A, C and E resulted in lower production of the above mentioned three byproducts, when compared to the control HiPDOG cultures (with no amino acid limitation) (**Figure 1A-C**). In the low amino acid HiPDOG cultures of CHO cell lines A, C and E, it was observed that butyrate accumulation was also lower when compared to control HiPDOG cultures (**Figure 1E-G**).This data suggested that amino acid catabolism could be contributing to butyrate formation in CHO HiPDOG cultures.

To assess if BCAA catabolism contributes to butyrate production, we analyzed the fed-batch cultures of CHO cell line D (WT) and the BCAT1 knock out (KO) clones derived from CHO cell line D. The KO clones lack the ability to catabolize BCAAs and therefore do not generate downstream intermediates and byproducts, namely isovalerate, isobutyrate and 2-methylbutyrate **(Figure 1D)** (Mulukutla et al., 2019). HiPDOG cultures of BCAT1 KO clones also produced butyrate at significantly lower rates as compared to the WT counterpart (**Figure 1H**). This established that catabolism of one or more of leucine, isoleucine or

valine was contributing to butyrate production by CHO cells in HiPDOG cultures. Interestingly, metabolomic analysis on spent medium from HiPDOG cultures of WT and BCAT1 KO clones showed that BCAT1 KO clones accumulated significantly lower levels of cholesterol, phosphatidylcholine, ceramides, and lysophospholipids (**Figure S2**). This suggested that BCAA catabolism contribution goes beyond SCFA biosynthesis to the overall fatty acid biosynthesis.

To probe which of the three branched chain amino acids contribute to butyrate production, CHO cell line C was cultured in fed-batch cultures in which leucine, isoleucine or valine were individually maintained at low concentration range, or all three amino acids were simultaneously maintained at lower concentrations, while keeping other amino acid levels at a typical (higher) concentration range **(Figure S3)**. A control fed-batch culture in which all the amino acids were maintained in the typical concentration range was also included. Cell-specific butyrate production rates ($q_{Butryate}$) were lower in conditions with lower levels of each of the three amino acids (**Figure 1I**) when compared to the control culture. The condition with all three BCAAs at lower concentration had the lowest $q_{Butryate}$ production. This established that catabolism of valine, isoleucine, and leucine all contribute to biosynthesis of butyrate in CHO cell cultures.

Butyrate, Isovalerate and Isobutyrate have q_P Enhancing Effect on Producing CHO Cell Lines

Butyrate, isovalerate and isobutyrate have been previously reported to be HDAC inhibitors (McBain et al., 1997). But, 2-methylbutyrate was reported to not have an impact on histone acetylation status and therefore was not evaluated further in this study for any potential q_P enhancing effect (H. J. Kim & Bae, 2011). Since butyrate is already known to have a q_P enhancing effect in some CHO cell lines (Jiang & Sharfstein, 2008), we wanted to investigate if isovalerate and isobutyrate also have q_P enhancing effect.

First, impact of reduced byproduct accumulation in fed-batch cultures on q_P was investigated. Cell line A was cultivated in HiPDOG cultures in a low amino acid condition (Low 8AA) or in a typical amino acid condition (Control). In addition to these two conditions, a third condition was also included where cell line A was cultivated in low amino acid condition but with supplementation of butyrate (1 mM) and isovalerate (4 mM) on Day 7 (Low 8 AA + SCFA). Reduced accumulation of isovalerate and butyrate was observed in the late stages of a Low 8AA condition. In addition, this condition also had lower q_P when compared to the Control condition. In the Low 8AA + SCFA condition, where butyrate and isovalerate were added back on Day 7, q_P was lower until Day 7 but increased over time after Day 7 reaching the level of Control condition by Day 10 (**Figure 2**). This data suggested that reduced production of isovalerate and butyrate could have a negative impact on q_P.

To confirm this phenomenon, a second experiment was performed using cultures taken from Day 6 of a Low 8AA fed-batch culture of cell line A. These cultures were treated with either 1 mM of butyrate, 5 mM of isovalerate, a combination of both or with no treatment (control) and cultivated in shake flasks for a period of 6 days in fed-batch mode. There was no growth difference observed between the conditions. The shake flask conditions treated with butyrate, isovalerate or combination of both had higher q_P and titers than the no treatment condition **(Figure S4)**. Subsequently, the q_P-enhancing effect of SCFAs was probed on a different cell line. In this case, cell line B was cultured in a bioreactor employing the HiPDOG process along with 10 amino acids being maintained in a lower concentration range (Low 10AA). The 10 amino acids included leucine, isoleucine and valine. On Day 7, cultures were taken from the bioreactor and transferred into 15-mL ambr® miniature bioreactors (see methods section for more details). These cultures were treated with various concentrations of isovalerate (2.5 or 5 mM), butyrate (1 or 2 mM) or isobutyrate (1 or 2 mM), individually, and cultivated in fed-batch mode for 5 days. An increase in the q_P was observed in all the treated conditions when compared to the

condition with no treatment **(Figure S5).** In addition to isovalerate and butyrate, isobutyrate also increased q_P, albeit to a much lower degree.

Next, the q_P enhancing effect of these byproducts on a BCAT1 KO clone (derived from cell line D) was probed. BCAT1 KO clones produce low or negligible amounts of these byproducts in fed-batch cultures. The BCAT1 KO clone was cultivated using the HiPDOG process in bioreactors and individually treated with 2mM of butyrate, a combination of 2.5 mM isovalerate, 1.5 mM isobutyrate and 0.3 mM butyrate, or with no treatment (control). Simultaneously, the wild type cell line D was also cultivated in bioreactors using the HiPDOG process with no treatment. As expected, the BCAT1 KO clone produced significantly lower levels of SCFAs in the untreated condition when compared to wild type cell line D. Further, BCAT1 KO clone had lower q_P compared to wild type cell line D (**Figure 3**). Interestingly, an increase in q_P was observed in BCAT1 KO conditions treated with either butyrate or the combination of SCFAs when compared to the BCAT1 KO condition with no treatment. Combined treatment increased q_P of the BCAT1 KO clone to levels similar to those observed in wild type cell line D. The above data establishes that the BCAA catabolic byproducts isovalerate, isobutyrate and butyrate can act as q_P enhancers in CHO cell cultures.

Rate of Production of SFCA Inversely Correlated to Lactate Production Rate

The glycolytic activity of mammalian cell lines including NS0 and CHO cells is known to change significantly over the span of a fed-batch culture (Le et al., 2012; Mulukutla et al., 2012; Mulukutla, Yongky, Grimm, Daoutidis, & Hu, 2015). Therefore, we next investigated if the production rate of SCFAs is impacted by glycolytic activity. HiPDOG technology offers a way to reduce glycolytic activity of CHO cells in fed-batch cultures by limiting the residual levels of glucose to lower concentration ranges (~0-0.1g/L) (Gagnon et al., 2011). We tested if the production rates of SCFA are different in fed-batch

cultures of CHO cells using HiPDOG technology when compared to non-glucose-limited fed-batch cultures where residual glucose levels are maintained at higher concentrations (>1.5 g/L).

CHO cell line A was cultured in a non-glucose-limiting fed-batch or a HiPDOG culture, with identical processes, besides the residual level of glucose in culture. The nutrient feed delivered was also similar between the two conditions on a per cell basis. For glycolytic activity, specific lactate production (q_{Lac}) was used as a metric. Specific short chain fatty acid production (q_{SCFA}), which is the combined rate of production of butyrate, isovalerate and isobutryate on a per cell basis, was also calculated for both conditions. As expected, the q_{Lac} was significantly lower in the HiPDOG culture when compared to the non-glucose-limiting fed-batch culture (**Figure 4A**). Interestingly, the q_{SCFA} was significantly higher in the HiPDOG culture when compared to the non-glucose-limiting fed-batch culture (**Figure 4C**). To confirm these results, a similar experiment was performed with a different cell line (cell line D); cell line D was cultivated in duplicates in a HiPDOG culture or a non-glucose-limiting fed-batch culture. The q_{Lac} was again significantly lower in the HiPDOG culture compared to the non-glucose-limiting fed-batch culture (**Figure 4B**). As observed in case of CHO cell line A, the q_{SCFA} was also significantly higher in the HiPDOG culture for cell line D when compared to the fed-batch culture (**Figure 4D**). Even across non-glucose-limiting fed-batch cultures, CHO cell cultures that metabolically shift to lactate consumption state due to lower glycolytic activity produce SCFA at higher rates than those that don't metabolically shift due to high glycolytic activity (data not shown). This suggests that SCFA production is negatively correlated with the glycolytic activity in CHO cell cultures.

Higher Accumulations of SCFAs in HiPDOG Cultures Results in Increased Specific Productivities

Next, accumulation levels of the SCFAs in culture milieu and cell specific productivities were probed for HiPDOG and non-glucose limiting fed-batch cultures for both cell line A and cell line D. HiPDOG cultures of both the cell lines had higher peak cell densities than the non-limiting fed-batch cultures (**Figure 4E**

and F). The higher densities combined with higher q_{SCFA} resulted in higher accumulation of SCFAs in the culture milieu of HiPDOG cultures when compared to non-glucose-limiting fed-batch cultures for both the cell lines (**Figure 4G and 4H**). As shown above (**Figure 2, 3, S4 and S5**), exposure to higher levels of SFCA results in higher qP. As expected, HiPDOG cultures exhibited higher qP across the course of the culture when compared to the non-limiting fed-batch cultures for both the cell lines (**Figure 4I and 4J**).

To confirm this phenomenon with another cell line, cell line G was similarly cultivated in a HiPDOG or a non-glucose limiting fed-batch cultures. All process parameters were kept constant besides glucose concentrations and feeding strategy. Similar viable cell densities (**Figure S6A**), and a robust lactate shift (**Figure S6B**) was seen in both the HiPDOG and the fed-batch conditions. However, there was higher titer in the HiPDOG conditions (**Figure S6C**), due to a higher q_P when compared to the fed-batch conditions (**Figure S6D**). To further support this concept, an additional four cell lines (cell lines H, I, J and K) were cultivated in HiPDOG or fed-batch cultures, where all parameters besides glucose and feed strategy were kept constant. The trend observed previously in cell lines A and G was also seen in cell lines H, I, J and K. In each case, the HiPDOG condition had a similar peak cell density to the fed batch condition (**Figure S7A-D**), but the HiPDOG condition had a higher q_P than the fed batch condition (**Figure S7E-H**).

Figure 5 shows a proposed mechanism for this phenomenon. In non-glucose limiting fed-batch cultures, high glycolytic flux lowers the production of short chain fatty acids from catabolism of branched chain amino acids, thus, resulting in lower accumulation of the same in culture yielding lower q_P. On the other hand, in the case of HiPDOG cultures, glycolytic fluxes are significantly lower resulting in higher production of short chain fatty acids. This leads to higher levels of accumulation in culture milieu and simultaneous increase in q_P.

Discussion

If there was a race to become the workhorse for recombinant protein production over the last two decades, CHO cells outright won the race leaving behind other alternative eukaryotic hosts including yeast cells, mouse myeloma, HEK 293 and insect cell lines. Some of the obvious reasons for this include CHO cell's ability to produce recombinant proteins with glycoform similar to those found in humans, the plasticity on offer in CHO cells that can be exploited to produce different types of proteins with varying product quality attributes, and the resilience of CHO cells to withstand harsh cell culture conditions typically observed in the late stages of fed-batch cultures. It appears that advantages for protein production seem to go beyond these well-established facets. Here, we show for the first time that CHO cells produce metabolic products that accumulate in culture milieu and act in an autocrine fashion as q_P enhancers i.e. enhance specific productivity. These byproducts include butyrate, isobutyrate and isovalerate. Accumulation of these byproducts in fed-batch processes can result in enhanced protein production, possibly in part explaining the higher q_p sometimes exhibited in fed-batch culture compared to highly proliferative continuous perfusion processes, in which such byproducts are continuously flushed from the bioreactor. Gagnon et al. showed that in a linked continuous-stirred-tank-reactor (CSTR) process using CHO cells, where N-1 CSTR operating at a higher perfection rate feeds cells into low perfusion rate N-stage CSTR, the N-stage CSTR has significantly higher q_P when compared to the N-1 CSTR (Gagnon, Nagre, Wang, Coffman, & Hiller, 2019). Authors show that N-stage CSTR has significantly higher accumulation of isovalerate in culture milieu when compared to the N-1 CSTR, which explains the higher q_P observed in N-stage CSTR.

Isovalerate and isobutyrate have been previously shown to be produced by catabolism of leucine and valine, respectively (Mulukutla et al., 2019). Butyrate, which is routinely used as a CHO cell specific productivity inducer, is thought to be a product of glucose fueled fatty acid synthesis. But, in this study,

we show data that suggests that branched chain amino acid catabolism as a key source of butyrate production in CHO cell fed-batch cultures. BCAA catabolism is known to generate intermediates of the TCA cycle. Some of these intermediates could be channeled towards fatty acid metabolism in CHO cells. Further, catabolism of other amino acids that generate TCA cycle intermediates could also be contributing towards butyrate production. More experimentation is needed to fully establish the complete list of nutrient sources for butyrate in CHO cell fed-batch cultures.

The mode of action of these byproducts to increase specific productivity could be through induction of epigenetic changes, specifically, by opening the chromatin into euchromatin state. Previous work in colon cancer cell lines established that supplementation of butyrate, isobutyrate or isovalerate result in increases in acetylation of histones (McBain et al., 1997). If the dominant copies of the product gene are present in the newly formed euchromatin region, it can lead to increase in product gene transcription potentially triggering enhanced protein synthesis. In a scenario where the dominant gene is already present in a euchromatin region prior to accumulation of byproducts, ensuing chromatin remodeling due to byproduct accumulation may not yield any increased protein production. In cases where transcription is not a bottleneck, any increase in the q_P observed due to supplementation of SCFAs could be due to the indirect effect of epigenetic changes on the secretory pathway components (Kantardjieff et al., 2010; Yee, de Leon Gatti, Philp, Yap, & Hu, 2008).

Interestingly, the production of these byproducts appears to be negatively linked to activity of glycolysis. Cells seem to produce higher levels of these byproducts either when glucose consumption rates are artificially lowered by limiting glucose supply, as in HiPDOG cultures. Further, producing CHO cell clones which intrinsically have lower glycolytic activity seem to produce more of these byproducts in fed-batch cultures when compared to the counterparts with higher glycolytic activity (data not shown). Such lower intrinsic glycolytic activity enables more potent metabolic shift to lactate consumption in the later stages

of fed-batch cultures. On similar lines, hybridoma continuous cultures with lower DL/DG (lactate produced/glucose consumed) had higher BCAA consumption rates (analogous to SCFA production rates) when compared to the continuous cultures of the same cell line with higher DL/DG (Europa, Gambhir, Fu, & Hu, 2000). The higher or lower DL/DG states were attained by initially cultivating the same cell line for a period of time in a batch mode with higher residual glucose levels or in a fed-batch mode with lower residual glucose levels, respectively, before switching them to continuous mode of operation. The underlying mechanism that governs this relationship between the two catabolic pathways, i.e. glycolysis and BCAA catabolism, has not yet been elucidated. It's possible that a drop in glycolytic activity can result in an increase in mitochondrial NAD^+/NADH ratio, which could increase mitochondrial BCKDH activity (Parker & Randle, 1978). As BCKDH is a rate limiting enzyme in the BCAA catabolism, increase in its activity could result in enhanced SCFA formation. More experimentation is needed to confirm this hypothesis.

The negative correlation between glycolysis activity and production of these byproducts also offers an explanation for the well-established correlation between metabolic shift to lactate consumption state and higher productivities in fed-batch cultures (Le et al., 2012), the root cause for which remains not fully understood. Cultures that undergo the metabolic shift, due to lower glycolytic rates, accumulate higher SCFA at higher rates which consequently enhances cell-specific productivity and increases overall productivity of the culture. The continuous increase in q_P sometimes observed in late stages of metabolic shifted cultures could be accredited to the autocrine response to the steady accumulation of these byproducts. Such understanding of the role of these novel byproducts can help contrive novel strategies for development of optimized cell culture processes.

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Tables

Compound	Source	ATCC CHO K1 Derived Cell Line Day 7 HiPDOG (n=2) (mM)	Cell Line A Day 10 HiPDOG (mM)	Cell Line B Day 10 HiPDOG (mM)	Cell Line C Day 10 HiPDOG (mM)	Cell Line D Day 10 HiPDOG (mM)	Cell Line E Day 7 HiPDOG (mM)	Cell Line A Day 12 Hybrid Perfusion Fedbatch (mM)
2-Methylbutyrate	Isoleucine	2.2	3.3	2.9	3.4	2.8	2.5	7.7
Butyrate	Glucose, BCAA	0.6	0.9	0.5	0.9	0.8	0.2	2.1
Isobutyrate	Valine	1.6	2.0	2.0	2.6	2.6	0.6	2.7
Isovalerate	Leucine	3.5	4.3	4.1	3.2	3.7	1.4	5.4
SCFA Sum	***N/A***	***7.9***	***10.5***	***9.5***	***10.1***	***9.9***	***4.5***	***17.0***

Table 1: Short chain fatty acid accumulation in fed-batch cultures of different CHO cell lines. The concentration of short chain fatty acids (2-methylbutyrate, isobutyrate, isovalerate and butyrate) measured in the extracellular milieu on day 7, day 10 or day 12 of fed-batch cultures of different CHO cell lines that were used in these studies. Data from a hybrid perfusion fed-batch culture for one of the cell lines is also included.

Figure Legends

Figure 1: SCFA and butyrate levels in multiple CHO cell line cultures with low amino acid process or with BCAT1 knocked out. Isovalerate (●), isobutyrate (▲), and 2-methylbutyrate (■) levels in extracellular milieu were measured over time in multiple cell lines with (dotted lines) or without (solid lines) amino acid limitation. A) SCFA levels in cell line A in low 8 amino acid (dotted line) and control (solid line) HiPDOG process. B) SCFA levels in cell line C in low 10 amino acid (dotted line) and control

(solid line) fed-batch process. C) SCFA levels in cell line E in low 10 amino acid (dotted line) and control (solid line) HiPDOG process. D) SCFA levels in cell line D in clone with BCAT1 gene knocked out (dotted line) and wild type (solid line) in a HiPDOG process. Butyrate levels in the extracellular milieu were also measured. E) Butyrate levels in cell line A in low 8 amino acid (dotted line) and control (solid line) HiPDOG process. F) Butyrate levels in cell line C in low 10 amino acid (dotted line) and control (solid line) fed-batch process. G) Butyrate levels in cell line E in Low 10 amino acid (dotted line) and control (solid line) HiPDOG process. H) Butyrate levels in cell line D in clone with BCAT1 gene knocked out (dotted line) and wild type (solid line) in a HiPDOG process.

Figure 2: q_P enhancing effect of butyrate and isovalerate on cell line A in low 8AA cultures. Cell line A was run in a control HiPDOG process (■ & solid line), a low 8 AA HiPDOG process (◆ & large checkered line), and a low 8 AA HiPDOG process with butyrate and isovalerate added back on day 7 (▲ & small dotted line). A) Viable cell densities of HiPDOG cultures. B) Extracellular leucine concentration for different conditions. C) Extracellular butyrate concentration for different cultures. D) Product of interest (POI) titer of different conditions. E) q_P for different conditions. F) Isovalerate concentration measured in extracellular milieu for different conditions.

Figure 3: q_P enhancing effect of butyrate, isovalerate, and isobutyrate on BCAT1 KO clone of cell line D. Cell line D and a BCAT1 KO clone derived from Cell Line D were cultivated in a HiPDOG process (1 condition for cell line D and three for BCAT1 KO). On Day 7 the BCAT1 KO clone conditions were individually treated with 2mM of butyrate (▲), a combination of 2.5 mM isovalerate, 1.5 mM isobutyate and 0.3 mM butyrate (●), or with no treatment (◆).Cell line D was cultivated with no treatment (■). A) Viable cell densities for different conditions. B) Relative culture isobutyrate/butyrate levels (arbitrary units). C) Relative culture isovalerate levels (arbitrary units). D) q_P for different conditions. E) POI titer for different conditions. F) Leucine concentration of different conditions

Figure 4: Cell specific production rate of SCFAs inversely linked to lactate production, and HiPDOG cultures produce SCFA at a higher rate and induce qP. Cell line A qLac (A) and qSCFA (C) in HiPDOG (dashed line) and fed-batch cultures (solid line). Cell line D qLac (C) and qSCFA (D) in HiPDOG and fed-batch cultures. Cell densities in the HiPDOG conditions were higher when compared to fedbatch for both cell line A (E) and D (F). Short chain fatty acid accumulation in the culture was significantly higher in the HiPDOG (dashed line) culture for cell line A (G) and cell line D (H) when compared to the control fed-batch cultures (solid line), due to a higher qSCFA seen in the HiPDOG conditions for both cell lines. The higher accumulation of SCFAs corresponded with an increase in qP in the HiPDOG cultures (dashed line) for cell line A (I) and D (J) when compared to the control fed-batch cultures (solid line) of the two cell lines.

Figure 5: A Mechanism Linking Glycolytic Activity to an Increase in q_P. A) In fed-batch cultures, high glycolytic flux lowers the production of short chain fatty acids from catabolism of branched chain amino acids. Thus, resulting in lower accumulation of the same in culture yielding lower qP. B) In HiPDOG cultures, glycolytic fluxes are significantly lower resulting in higher production of short chain fatty acids. This leads to higher levels of accumulation in culture milieu and corresponding increased q_P.

Figure S1: Levels of branched chain amino acids and viable cell densities of cultures in low amino acid and control conditions from Figure 1. Isoleucine (■), leucine (●), and valine (▲) levels in the extracellular milieu were measured in both low amino acid (dotted line) and control (solid line) processes for A) cell line A, B) cell line C and C) cell line E in the same experiments from Figure 1. Isoleucine (■), leucine (●), and valine (▲) levels in the extracellular milieu were also measured in a D) BCAT1 knock out clone (dotted line) and wild type clone (solid line) for cell line D in the same experiment from Figure 1. Viable cell densities are also shown with low amino acid (dotted line) and

control (solid line) processes for E) cell line A, F) cell line C, and G) cell line E in the same experiments from Figure 1. H) Shows BCAT1 knock out (dotted line) and wild type clone (solid line) for cell line D.

Figure S2: Relative culture milieu accumulations of cholesterol, phosphatidylcholine, ceramides, and lysophospholipids in HiPDOG cultures of WT and BCAT1 KO clone of cell line D. Extracellular milieu concentrations showed that the BCAT1 KO clone (green lines) accumulated significantly lower levels of cholesterol, phosphatidylcholine, ceramides, and lysophospholipids in spite of higher peak cell densities when compared to WT cell line (blue lines).

Figure S3: Levels of branched chain amino acids and lysine in cultures of Figure 1I. Supernatant concentrations for A) isoleucine, B) valine, C) leucine and D) lysine for control (■, solid line), low isoleucine (◆, small dotted line), low valine (●, dashed line), low leucine (▲, small checkered line) and low 3 BCAA (□, checkered line) conditions.

Figure S4: q_P enhancing effect of butyrate and isovalerate individually and synergistically on cell line A in low 8AA cultures. Aliquots of cell line A culture from a low 8AA HiPDOG bioreactor was transferred to shake flasks on day 6 and were cultivated in fed-batch mode from day 6 through day 14. On day 6, the shake flasks were supplemented with either 5 mM isovalerate (●), 1 mM butyrate (◆), neither (used as a control) (■) or 5 mM isovalerate and 1 mM butyrate added in combination (▲). A) Viable cell densities for different conditions. B) q_P for different conditions. C) POI titer for different conditions

Figure S5: qP enhancing effect of butyrate, isovalerate and isobutyrate at different concentrations on cell line B cultivated in low 10AA HiPDOG cultures. On day 7, these cultures were treated with different concentrations of isovalerate at 2.5 mM (◆) or 5 mM (●) and the qP (A), titer (D) and VCD (G) was compared with two control conditions with no additives (▲, ■). Alternatively, cultures were treated

with different concentrations of butyrate at 1 mM (◆) or 2 mM (●) and the qP (B), titer (E) and VCD (H) was compared with two control conditions with no additives (▲, ■). Thirdly, cultures were treated with different concentrations of isobutyrate at 1 (◆) mM or 2 mM (●) and the qP (C), titer (F) and VCD (I) was compared with two control conditions with no additives (▲, ■).

Figure S6: Cell line G has a higher q_P when cultivated in HiPDOG cultures when compared to traditional fed-batch cultures. Cell line G was cultivated in a HiPDOG (▲) or a non-glucose-limiting fed-batch (●) cultures. All process parameters were kept constant besides residual glucose concentration. A) Similar viable cell densities were seen between cultures. B) A robust lactate shift was seen in both the HiPDOG conditions and the traditional fed-batch conditions. C) Higher titer of the POI observed in the HiPDOG conditions. D) A higher q_P was seen in the HiPDOG cultures when compared to the fed-batch cultures.

Figure S7: Cell line H, cell line I, cell line J, and cell line K have increased q_P in HiPDOG cultures when compared to traditional fed-batch cultures. An additional four CHO cell lines were cultivated in HiPDOG (▲) or fed-batch cultures (●). A) Cell line H viable cell densities and q_P. B) Cell line I viable cell densities and q_P. C) Cell line J viable cell densities and q_P. D) Cell line K viable cell densities and q_P.