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# Addressing amino acid-derived inhibitory metabolites and enhancing CHO cell culture performance through DOE-guided media modifications

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## Abstract

Previously, we identified six inhibitory metabolites (IMs) accumulating in Chinese hamster ovary (CHO) cultures using AMBIC 1.0 community reference medium that negatively impacted culture performance. The goal of the current study was to modify the medium to control IM accumulation through design of experiments (DOE). Initial over-supplementation of precursor amino acids (AAs) by 100% to 200% in the culture medium revealed positive correlations between initial AA concentrations and IM levels. A screening design identified 5 AA targets, Lys, Ile, Trp, Leu, Arg, as key contributors to IMs. Response surface design analysis was used to reduce initial AA levels between 13% and 33%, and these were then evaluated in batch and fed-batch cultures. Lowering AAs in basal and feed medium and reducing feed rate from 10% to 5% reduced inhibitory metabolites HICA and NAP by up to 50%, MSA by 30%, and CMP by 15%. These reductions were accompanied by a 13% to 40% improvement in peak viable cell densities and 7% to 50% enhancement in IgG production in batch and fed-batch processes, respectively. This study demonstrates the value of tuning specific AA levels in reference basal and feed media using statistical design methodologies to lower problematic IMs.

**KEYWORDS**
amino acid metabolism, Chinese hamster ovary, design of experiments, fed-batch bioprocessing, inhibitory metabolites, medium optimization

## 1 | INTRODUCTION

The market of biotherapeutics has progressed significantly since the approval of the first monoclonal antibody by the United States Food and Drug Administration (US FDA) in 1986 (Liu, 2014). Antibodies and other therapeutic proteins represent some of the best-selling drugs in the pharmaceutical market with 8 of the top 10 drugs being biologics in 2018 (Lindsley, 2019; Lu et al., 2020). Given the industrial importance of these biologics, there is a continuing need to understand existing biomanufacturing practices as well as to develop and implement methods that improve their yield and productivity. Chinese Hamster Ovary (CHO) cells are the workhorses of the

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biopharmaceutical industry due to their capacity to produce human-compatible monoclonal antibodies, bispecific antibodies, and other biotherapeutics. Their ability to grow in suspension cultures and ease of scale-up, capability to perform complex posttranslational modifications including human-like glycosylation, and lower susceptibility to viral contaminations are some of the major factors leading to the choice of CHO cells for bioproduction application (Dhara et al., 2018). One of the critical aspects of bioprocess development for CHO cell cultures is the design and implementation of highly efficient biomanufacturing processes in which the nutrient requirements of the mammalian cells are identified and satisfied to ensure efficient growth and production of these valuable biologics.

CHO cells in culture grow and produce therapeutic protein of interest by relying on the nutrients provided which are processed within their metabolic networks. These nutrients include sugars, amino acids, and other less abundant nutrients and cofactors utilized by the cells for growth, maintenance, and protein production. Robust cell growth, elevated viable cell densities, and high product titers can be achieved through proper utilization of glucose and amino acids and their efficient/effective conversion into energy, biomass, and bioproducts. Unfortunately, the disposition of nutrients to energy or useful metabolites does not always occur in cell culture processes (Coulet et al., 2022; Ritacco et al., 2018; Young, 2013). Alternatively, metabolic inefficiencies are manifested in part as the formation of pathway intermediates and their accumulation and secretion as by-products in cell culture over time (Pereira et al., 2018). Further, recent studies indicate that some of these metabolic by-products can be toxic to the cells, impacting either cell growth or protein productivity or both (Mulukutla et al., 2017). The toxicity of these secreted metabolites can be compounded when processes demand high cell densities present in fed-batch cultures. A potential consequence of this accumulation of inhibitory metabolites is the presence of a cell growth ceiling which in turn can limit the production capacity of the fed-batch process. This limitation makes growth inhibitory metabolite identification and characterization in CHO cell cultures an important step in process development.

These metabolites can emerge not just from central metabolism contributing to energy generation in CHO cells but also from inefficient utilization of amino acids and other nutrient inputs. Incomplete amino acid catabolic pathways have been shown previously to lead to the buildup of the pathway intermediates or dead-end pathway metabolites in the culture broth surrounding the cells (Mulukutla et al., 2017). However, if the levels of these metabolites secreted into the medium can be controlled, a cleaner and more robust bioprocessing environment is possible with a minimum of waste inhibitory metabolites both intracellularly and extracellularly. Efforts to control toxic CHO cell metabolites during bioprocessing have focused primarily on two approaches: either modifying the basal and/or feed medium or genetic manipulation to alter intracellular metabolism. For example, controlling glucose concentrations and maintaining it at a reduced level throughout the fed-batch culture using HiPDOG or other methods can successfully lower the amount of lactate accumulating into the culture medium in comparison to a traditional fed-batch process (Gagnon et al., 2011). This technique also resulted in higher growth and enhanced productivity of the cells as well. Additionally, the catabolic by-products of tyrosine, phenylalanine, and tryptophan, which can be highly toxic to CHO cell fed-batch cultures, were reduced by lowering the source amino acid concentration by half in the basal and feed medium (Mulukutla et al., 2017). Such efforts to manipulate the basal and feed medium components can be especially beneficial in optimizing media design for high-density cultures as part of process intensification. Furthermore, cellular metabolism can be engineered so that substrates such as glucose and amino acids are directed away from problematic metabolites that lead to dead-ends, off pathways metabolites, or accumulating intermediates and into pathways that yield more effective conversion into useful downstream metabolites. For instance, genetic intervention has been implemented using siRNA to knock down or CRISPR-Cas9 to knockout upstream genes such as BCAT1 in the branched-chain amino acid (BCAA) catabolic pathway to reduce the accumulation of toxic metabolites (Mulukutla et al., 2019).

Recently, our group applied LC-MS based metabolomics on fed-batch cell culture supernatant to identify a series of metabolites secreted by CHO cells namely, CMP, NAP, ACA, TRI, HICA, and MSA (Table 1). Furthermore, several of these metabolites, found to be inhibitory to CHO cell growth and protein production, are linked to the catabolism of amino acids (Kuang et al., 2021). Controlling these inhibitory metabolites by single amino acid precursors was hypothesized to ignore the combinatorial effects arising from intertwined network of amino acid metabolism. Thus, in the present study, medium modification strategies were implemented with the help of design of experiments (DOE) that allows us to adjust multiple amino acid precursors to reduce or at least limit these inhibitory metabolites. Such a rational medium design helps determine if a more effective fed-batch protein production process could be developed in consideration of these relationships. Design of experiments has often been used in cases where multiple factors need to be screened and further optimized for achieving the desired output (Bezerra et al., 2008). The availability of multiple options in a DOE framework offers the advantage of screening from a larger precursor set with a lesser number of experiments (González-Leal et al., 2011). In the context of medium/feed optimization for cell culture applications, a

**TABLE 1** List of inhibitory metabolites identified previously and considered for control strategy in the study.

| S. No. | Abbreviation | Name |
|---|---|---|
| 1 | CMP | Cytidine monophosphate |
| 2 | NAP | N-Acetylputrescinium |
| 3 | ACA | Aconitic acid |
| 4 | TRI | Trigonelline |
| 5 | HICA | 2-Hydroxyisocaproic acid |
| 6 | MSA | Methyl succinic acid |

cell growth and product titer focused DOE has been employed successfully in previous studies (Castro et al., 1992; Rafigh et al., 2014; Torkashvand et al., 2015). Torkashvand et al. reported a DOE methodology to design an amino acid feed medium to enhance the production yields of bevacizumab which also led to improvements in product quality attributes. Rafigh et al. utilized RSM and ANN methods to optimize six process variables of batch fermentation culture. Therefore, a DOE-driven medium manipulation approach was employed in the current study to examine our capacity to reduce the accumulation of some of these toxic by-products in CHO cell cultures. We have further shown that this method can be used to successfully lower inhibitory metabolite accumulation levels in fed-batch cultures by using only the metabolite secretion levels as the output parameters in which significantly contributing substrates are the input variables.

Initially, an experimental pathway analysis was conducted to determine the precursor matrix of substrates that contribute to the inhibitory metabolite accumulation to allow for significant factor screening. This also enabled the elimination of a subset of amino acids precursors that likely do not contribute to the accumulation of these target metabolites and thus need not be optimized. Next, the resulting design space was subjected to a two-level DOE study with component screening initially followed by optimization of screened components from the design space. The first outcome of the two-level DOE study was to reduce the accumulated levels of inhibitory metabolites that are potentially problematic for cell growth and product titers in fed batch. The resulting amino acid levels from the DOE analysis were then translated into a modified basal medium and feed medium formulation. Finally, a validation study was conducted using the modified basal/feed media formulation to determine the impact on culture performance, in terms of cell growth, product yields, and inhibitory metabolite levels. Overall, we show that rational CHO culture media design with the objective focused to lower the accumulation of inhibitory metabolites can enhance the fed-batch culture performance of mammalian cells for bioproduction applications.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell line and subculturing

The cell lines used was a suspension-adapted CHO-K1 cell line producing IgG protein that was previously used in the metabolite identification and characterization study. The growth medium (AMBIC 1.0 reference community basal medium) for the cultures was obtained from Millipore Sigma (St. Louis, herein referred to as medium A) and was supplemented with 8 mM Glutamine (Corning) (Cordova et al., 2023). Cells were thawed into 30 mL of medium A with 8 mM glutamine in 125 mL plain-bottomed shake flasks (Fisherbrand) in a humidified incubator (Multitron Infors HT), at 37°C and 5% $CO_2$ at 125 rpm. Cells were subcultured every 3–4 days when they reached a cell density of $>3 \times 10^6$ cells/mL. The cell viability was maintained above 98% at every subculturing stage.

### 2.2 | Experimental plan

A two-stage experimental plan was implemented to design a control strategy for inhibitory metabolites. First, a precursor amino acids matrix contributing to the accumulation of these metabolites was constructed (Precursor study). This was done initially by understanding the amino acid consumption profiles. Then, metabolite accumulation levels were evaluated by the over-supplementation of potential precursor amino acids. Subsequently, the precursor amino acids identified in the "Precursor study" were optimized using a design of experiments methodology towards reduced metabolite accumulation (Design study). The resulting optimal amino acid levels were finally tested against a control process where these metabolites were observed to be accumulating at high concentrations.

### 2.3 | Batch and fed-batch process

For a control batch process, cells were inoculated in medium A supplemented with 8 mM Glutamine at a seeding cell density of $0.5 \times 10^6$ cells/mL in a working volume of 30 mL. This study was conducted in biological duplicates and daily cell culture samples were collected for growth measurement and extracellular metabolite analysis. Cultures were harvested when the cell viability dropped below 70%. An amino acid depleted version of medium A was kindly provided by Millipore Sigma (medium A−) and was used for DOE studies described in Section 3.3.

For a control fed-batch process, cells were inoculated in medium A supplemented with 8 mM Glutamine at a seeding cell density of $0.5 \times 10^6$ cells/mL in a working volume of 30 mL. Starting Day 3, a nutrient-rich feed medium B (AMBIC 1.0 reference community basal medium) was added daily to the CHO cell cultures at a rate of 10% (v/v) (Cordova et al., 2023). This study was conducted in biological duplicates and daily cell culture samples were collected for growth measurement and extracellular metabolite analysis. Glucose was supplemented up to 5 g/L to the culture when its concentration dropped below 2 g/L. Cultures were harvested when the cell viability dropped below 70%. An amino acid depleted version of feed B was kindly provided by Millipore Sigma (feed B−) and was used for DOE studies described in Section 3.3.

### 2.4 | Cell growth, viability, and productivity determination

Cell culture samples were collected every 24 h for estimation of the culture performance at various stages of the medium design process. Cell growth was determined through viable cell density (VCD) measurement using a hemocytometer. Also, culture viability was measured daily using the trypan blue dye exclusion method. Cell growth rate was calculated by fitting the VCD data to an exponential function. Titer analysis was also performed via HPLC using a protein A column (Poros 2 µm, 2.1 × 30 mm; Thermofisher). To compare and

evaluate the productivity of the cells in optimized media and feed, specific titer productivity was calculated which is defined as the overall measured titer (mg/L) normalized against the respective integral viable cell density (IVCD).

## 2.5 | Glucose, lactate, and amino acids measurements

Concentrations of glucose and lactate were measured by YSI 2950 biochemistry analyzer (YSI). Cell culture supernatant collected every 24 h of control batch cell culture were used to measure the amino acid concentrations for understanding the consumption dynamics of various amino acids. Concentrations of alanine, glycine, valine, leucine, isoleucine, proline, methionine, serine, threonine, phenylalanine, aspartate, asparagine, glutamate, glutamine, lysine, glutamine, arginine, and tyrosine were measured using a GC-MS based method used in previous studies (Woo Suk & Antoniewicz, 2013). Fifteen microliter of a standard solution containing [U-$^{13}$C] algal amino acids and [U-$^{13}$C] glutamine was mixed with 15 µL of spent medium sample. This 30 µL mixture was dried at room temperature under nitrogen gas flow using an evaporator (Reacti-Vap/Reacti-Therm). Next, 35 µL of 2 wt% methoxylaminehydrochloride (MOX) in pyridine solution was added and samples were incubated at 37°C for 90 min. Finally, 70 µL of N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) + 1% tert-butyldimetheylchlorosilane (TBDMCS) (Thermo Scientific) was added and samples were incubated at 60°C for 30 min. After the end of incubation period, samples were centrifuged at 14000 rpm for 5 min and 50 µL of the top clear solution was transferred to the GC vial for injection.

GC/MS analysis was performed on an Agilent 7890A GC system equipped with a DB-5MS capillary column (30 m, 0.25 mm i.d., 0.25 µm-phase thickness; Agilent J&W Scientific), connected to an Agilent 5977B Mass Spectrometer operating under ionization by electron impact (EI) at 70 eV. Helium flow was maintained at 1 mL/min. The source temperature was maintained at 230°C, the MS quad temperature at 150°C, the interface temperature at 280°C, and the inlet temperature at 280°C. For GC/MS analysis of amino acids as well as polar metabolites, 1 µL was injected at a split ratio based on the peak intensities. The column was started at 80°C for 2 min, increased to 280°C at 7°C/min, and held for 20 min bringing the total run-time for one sample to 50.33 min. Mass spectra were recorded in single ion monitoring (SIM) mode with 4ms dwell time on each ion. Mass isotopomer distributions were obtained by integration of ion chromatograms and corrected for natural isotope abundances (Antoniewicz et al., 2007).

## 2.6 | Inhibitory metabolite measurements

Standard solution containing 20 mM trans-aconitic acid (ACA), 320 µM leucinic acid (HCA), 64 µM methyl succinic acid (MSA), 16 µM indole-3-carboxylic acid (ICA), 64 µM cytidine monophosphate (CMP), 64 µM guanosine monophosphate (GMP), 3.2 µM trigonelline (TRI) and 3.2 µM N-acetyl putrescine (NAP) in water was prepared, followed by serial dilution with water to prepare working standard solutions. An internal standard (IS) solution containing 500 µM 13C6-trans-aconitic acid, 200 µM 2H3-leucinic acid, 20 µM 2H6-methyl succinic acid, 50 µM 2H5-indole-3-carboxylic acid, 50 µM 15N3-cytidine monophosphate, 50 µM 15N5-guanosine monophosphate, 0.5 µM 2H3-trigonelline and 5 µM 2H3-N-acetyl putrescine was prepared in water. Standard calibration samples were obtained by mixing 10 µL of (blank) culture medium, 5 µL of the IS solution, 5 µL of each working standard solution and 80 µL of acetonitrile with 1% formic acid. After vortex for 30 s, the samples were centrifuged (16,000*g*–10 min) and the supernatant was collected for LC/MSMS analysis. Study samples were prepared by mixing 10 µL of cell culture sample, 5 µL of the IS solution, 5 µL of water, and 80 µL of acetonitrile with 1% formic acid, followed by vortex, centrifugation, and collecting the supernatant for analysis. Samples were analyzed in triplicate measurements with Thermo Scientific Ultimate 3000 HPLC system coupled with a Thermo Scientific TSQ Quantiva triple quadrupole mass spectrometer. Samples were injected at 5 µL volume. Chromatographic separation was achieved on a SeQuant ZIC-cHILIC column (2.1 × 100 mm, 3 µm) with ZIC-cHILIC guard column (2.1 × 20 mm, 5 µm).

## 2.7 | Statistical design of experiments

### 2.7.1 | Screening design

Significant factor screening of the precursor matrix was conducted using a randomized screening design implemented with JMP Pro 14. This was done to shortlist the AA factors that play a major role in the buildup of IMs in the culture. The set of factors correspond to the various AAs contributing to the accumulation of IMs as derived from a precursor study (Section 3.2). To construct the design matrix, an adaptation of the Plackett–Burman screening design was used, to determine the number of randomized experiments needed for screening. Typically, to screen for the main effect from n factors on the response variable, n + 1 experiments are required to be constructed. Each factor was tested at two different levels, high (+1) and low (−1), where the (+1) represented the basal medium concentration of the factor and (−1) represented the permissible lower bound for the factor as determined by the cell metabolism. To capture the statistical variance of the screening design, a dummy variable was introduced as a factor into the design matrix. The levels (+1) and (−1) of this dummy variable were kept unchanged. Thus, using a total of 11 factors, 12 randomized experiments were conducted in duplicates using CHO cells culture in batch mode. The response variable for the study was the IM concentration present at culture harvest. Figure 4 shows the design matrix along with the high and low levels of the various factors. Data was analyzed using the JMP Pro 14 software and the significant factors were selected for optimization using response surface design.

#### 2.7.2 | Response surface design

After selection of the significant amino acid factors using the screening design, the concentrations of these amino acids in the basal medium were optimized further using a Box–Behnken design. Box–Behnken design, a type of response surface design, employs testing of each factor at three levels to determine their impact on the response variable. Thus, each factor was tested at three different levels, (+1), (0), and (−1). While (+1) and (−1) correspond to the same concentrations of the factor that were tested in the screening design, an additional intermediate level (0) was added for testing as a center point level for each factor. The design matrix for the response surface design was constructed using the DOE toolbox available in JMP Pro 14 software. Each condition was tested in duplicates using CHO cells cultured in batch mode. The concentration of each toxic metabolite in the harvest culture was used as the response variable. Analyzing the design using linear least squares regression using JMP provided critical values of amino acid levels (considered in the design space) corresponding to each inhibitory metabolite. The critical value solution refers to the point in the design space where the derivative of the response equation is zero. The optimal solution for each amino acid required to minimize the concentration of the IMs in culture supernatant were determined by tabulating all the results and using physiological reasoning to support the decision.

#### 2.7.3 | Implementation study

The optimal concentrations of the significant AAs determined by the response surface design were used to reconstruct the basal and feed medium to create "reduced medium A" and "reduced feed B" for CHO cell cultures. This was done to characterize the improvements that the optimal basal and feed medium provided to the cell culture performance in batch and fed-batch mode. These improved batch and fed-batch processes were compared to the control processes (described in Section 2.3). The design study and the derived optimal amino acid concentrations were considered to be validated if these improved cultures provided cell growth, culture viability and/or product titer enhancements and most importantly, reduced the IM accumulation level in comparison to control cultures.

## 3 | RESULTS AND DISCUSSION

### 3.1 | Study overview

This rational medium design study was constructed in two parts such that the output of the first part (precursor study) served as the input for the second part (design study) as shown in Figure 1a. Within the precursor study, a set of potentially contributing substrate amino acids were first identified. Next, the design study served the task of screening these amino acids to shortlist the significant ones that led to higher accumulations of IMs. Once screened, the levels of this subset of amino acids were modified to lower concentrations in the basal and feed medium formulations. Finally, fed-batch experiments were conducted to finalize the basal and feed conditions that resulted in the highest growth and protein production.

### 3.2 | Precursor study

The first step was to determine which amino acid substrates act as precursors into metabolic pathways that result in the accumulation of inhibitory metabolites. Previously, we determined how some of these amino acids can map to their respective by-products through analysis of CHO metabolic pathways available in the KEGG database (Kanehisa, 2000). However, cellular metabolism is a complex network of multiple interwoven reactions wherein each metabolite can emerge from multiple substrates. This makes it difficult to pinpoint a specific source leading to the accumulation of a particular metabolite. This motivated us to evaluate the effects that result from a combination of multiple substrates leading to accumulation of different inhibitory metabolites. As a result, a precursor study was undertaken to identify which individual, and combination of substrates should be considered and manipulated in the culture medium for improved growth and productivity. A simplified decision tree for the precursor study described in Figure 1b summarizes the various tasks leading up to the selection of precursor amino acids for the design study.

#### 3.2.1 | Cell growth and amino acid consumption in batch culture

To evaluate the amino acid consumption rates, a control batch growth study was performed (Supporting Information: Figure S1). The batch process ran for 7 days during which samples were collected every 24 h for measuring the viable cell density (VCD) and monitoring the metabolite levels. As shown in Supporting Information: Figure S1a,b, cell viability remained above 95% percent until Day 5 during which the cells attained a maximum cell density of $12.7 \times 10^6$ cells/mL. Cell growth stopped after day 5 together with a sharp decline in cell viability to less than 80% on Day 6 and 0% on Day 7. The end of cell growth correlated with the depletion of central carbon sources, namely glucose at Day 5 and lactate at Day 6 (Supporting Information: Figure S1c,d). While glucose was initially the primary source of carbon for the CHO cells accompanied by an accumulation of lactate, there was a shift towards lactate utilization starting from Day 4. This continued until both the nutrients in the culture were completely exhausted leading to the stopping of cell growth and subsequently cell death.

Typically, commercial media are designed to sustain growth for a wide range of CHO cell lines and thus contain relatively standard amounts of different nutrients utilized as part of normal cellular metabolism. Knowledge about the amino acid concentrations over the duration of the batch culture will provide us with useful

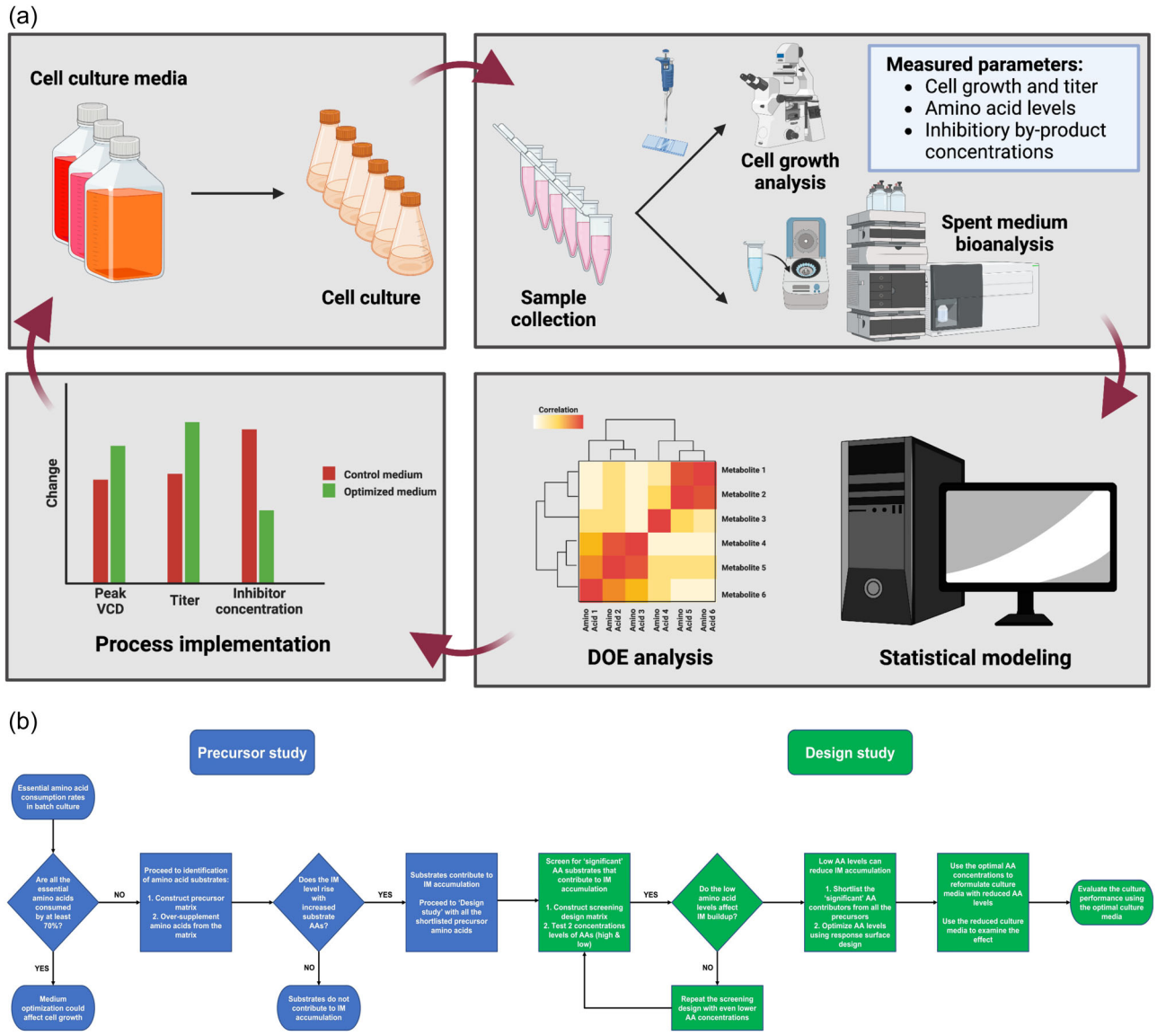



**FIGURE 1** (a) Medium design guided by statistical design of experiments, (b) process flowchart for study of the amino acid precursors that contribute to the accumulation of inhibitory metabolites followed by the design of experiments approach to determine the optimal amino acid levels to enhance CHO cell performance.

information about amino acid consumption patterns of the CHO cells in culture (Dhara et al., 2023). Therefore, shown in Figure 2 is a heat map profile of relative levels of amino acids in CHO cells (normalized to 100%) over time for a batch cell culture process using medium A supplemented with 6 mM L-Glutamine. Eighteen AAs (except Trp and Cys) out of which nine were nonessential (Figures 2a) and nine were essential (Figure 2b) were measured using a GC-MS. A contrasting difference was seen in the consumption patterns of nonessential amino acids (NEAAs) and essential amino acids (EAAs). Overall, five (Gln, Asn, Asp, Ser, and Pro) out of eight of the measured NEAAs were reduced to less than 30% or more of the starting levels while three others (Glu, Ala, and Gly) accumulated over the culture period. Glutamate is derived from glutamine metabolism (for most non-glutamine synthetase mammalian cell lines as used here). Glutamine, typically the second-most preferred carbon source in the CHO-K1 cell line, was completely exhausted in this batch study (Nicolae et al., 2014). Glutamine also, directly and indirectly, contributes to the biosynthesis of multiple other NEAAs including Glu, Asn, Ala, Asp, Pro, and Ser (Grinde et al., 2019; Kalhan & Hanson, 2012; Yoo et al., 2020). Asparagine was completely exhausted during the culture as well, while more than 75% aspartate in the media was also consumed. The high levels of serine consumption may be related to its interconversion into glycine inside the cells. Alternatively, the accumulation of Gly and Ala may be due to their formation as by-products of glycolysis while Ala may also form as a result of transamination reactions. Lastly, 60% of initially present tyrosine was gradually consumed by Day 6. Previous studies have shown that tyrosine plays a fundamental role in CHO cell biomass production

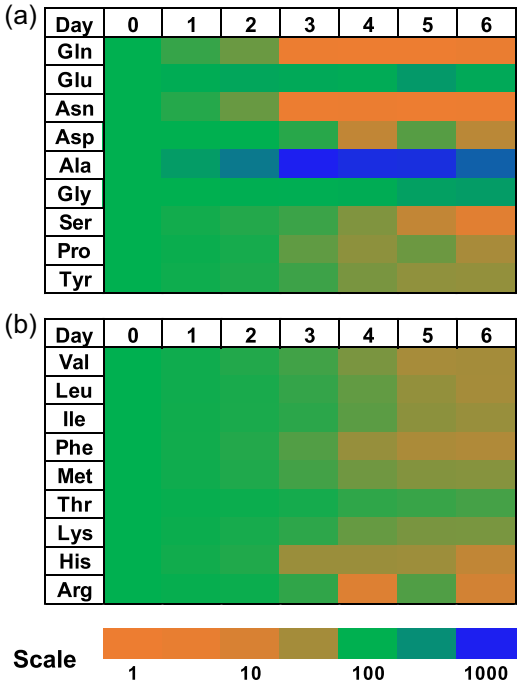



**FIGURE 2** Relative amino acid (AAs) levels measured in the culture medium for CHO-K1 cells when cultured in batch mode. The starting concentrations of the AAs have been designated as 100% (as measured on Day 0) and relative levels have been shown in the heat map. (a) Nonessential amino acids and (b) essential amino acids. Scale shown in the heat map refers to 1% of amino acid level in culture from left end (orange) to 1000% of amino acid level toward the right end (blue). $n = 2$ (refer to Supporting Information: Table S1A and S1B for numerical data).

and limitations in supply can be detrimental to cell growth (Traustason 2019).

Alternatively, a more gradual decline was observed for the EAAs (Figure 2b) during the process with 6 of them remaining in the spent medium at more than 30% of the starting concentration. This observation points to the possibility of improving the culture medium formulation by lowering the levels of some of these incompletely utilized amino acids without potentially impacting cell growth. Previous studies have shown that catabolism of essential amino acids can lead to the formation of growth inhibitory metabolic by-products (Ley et al., 2019; Mulukutla et al., 2017). These by-products can also be secreted by the cells into the extracellular environment and then exert an inhibiting impact on cell performance. Therefore, as a next step in the precursor study, we sought to elucidate amino acids linked to the accumulating inhibitory metabolites we identified in a previous study (Kuang et al., 2021).

### 3.2.2 | Identification of precursor amino acids

Previously, we identified 6 toxic metabolites, namely CMP, NAP, ACA, TRI, HICA, and MSA, listed in Table 1 that are inhibitory to growth of CHO cells in culture (Kuang et al., 2021) The accumulation of metabolic by-products is governed not just by their primary amino acid source, but also by other potentially interacting amino acids that feed into particular pathways. A list of primary and secondary substrates for these six toxic metabolites was compiled on the basis of whether or not they feed into a specific inhibitory metabolite (Figure 3a). In addition to these specific amino acid sources, other amino acids were added to the list of contributing amino acids as secondary substrates based on their potential to contribute into the pathway though other metabolites. For example, CMP and ACA are derived from the purine and pyrimidine metabolic pathway which predominantly has glucose as the contributing substrate with glutamine being the next dominant source. We added arginine, alanine, and asparagine to the substrate set since they can indirectly contribute to the accumulation of CMP and ACA (Brosnan & Brosnan, 2007; Hoang et al., 2022; Kim et al., 2011; Kuang et al., 2022; Saas et al., 2000). In addition to these precursors, KEGG pathway analysis suggested that proline could indirectly contribute to the accumulation of N-acetyl putrescine (NAP) through the generation of ornithine and putrescine. Trigonelline (TRI) is a part of the nicotinamide metabolism in cells, and we found that some major contributing precursors feeding toward trigonelline include amino acids such as tryptophan, aspartate, alanine, glutamine, and asparagine. Lastly, pathway intermediates arising from branched-chain amino acid catabolism including isoleucine and leucine led to the secretion of methyl succinic acid (MSA) and 2-hydroxyisocaproic acid (HICA), respectively, and were thus, added to the precursor matrix.

To confirm the curated precursor matrix, a preliminary fed-batch study was conducted by supplementing an excess of the potential contributing substrates in the basal medium that helped determine if these amino acids altered the accumulation of the corresponding toxic metabolites. The basal medium was constructed by modifying the relevant amino acids in medium A (basal medium) to double (2x-AA) and triple (3x-AA) the initial basal medium concentrations as shown in Figure 3a. For instance, to evaluate the impact of alanine, arginine, asparagine, and glutamine on the accumulation of CMP, medium A was modified such that all these amino acids were added at twice and thrice the concentration in the original basal medium A formulation. A fed-batch process consisting of original medium A amino acid concentrations with feed B (feed medium) as feed at a rate of 10% (v/v) starting at Day 3 was used as the experimental control. The feed medium B, its composition, and feed rate were not altered for these initial test cases. The metabolite concentrations were determined in comparison to control to evaluate the impact of the increased amino acid substrate levels in the basal medium A. The normalized inhibitory metabolite levels accumulated for all three scenarios on Day 12 are shown in Figure 3b (Control: blue, 2x-AA: yellow; 3x-AA: red).

When inhibitory metabolite levels were measured on Day 12, sharp increases in the levels of CMP, ACA, TRI, and HICA were observed in 3x-AA conditions with moderate increases in 2x-AA in comparison to control (Figure 3b). ACA accumulation was the greatest of the measured metabolites, with more than 3- and 15-fold increases in 2x-AA and 3x-AA conditions, respectively. CMP

(a)

| | CMP | NAP | ACA | TRI | HICA | MSA |
|---|---|---|---|---|---|---|
| Alanine | | | | | | |
| Arginine | | | | | | |
| Asparagine | | | | | | |
| Aspartate | | | | | | |
| Glutamine | | | | | | |
| Isoleucine | | | | | | |
| Leucine | | | | | | |
| Lysine | | | | | | |
| Proline | | | | | | |
| Serine | | | | | | |
| Tryptophan | | | | | | |

(b)

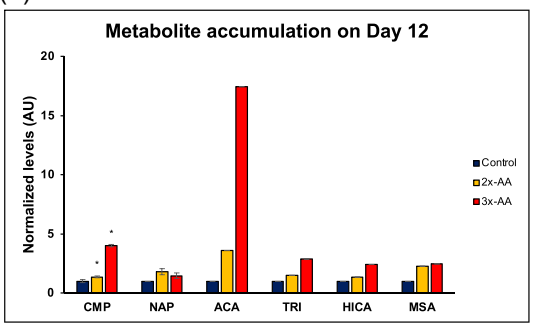



**FIGURE 3** Design and results of experimental pathway analysis for lumped source identification of metabolites in fed-batch cultures. (a) Amino acid substrate precursor matrix for each inhibitory metabolite and (b) inhibitory metabolite levels in cell culture supernatants on day 12 of fed-batch cultures. Mean ± SD; $n = 2$, $^*p < 0.1$. Statistics by unpaired two-tailed student $t$ test against control.

showed a 4-fold increase at 3x-AA and TRI, HICA, and MSA exhibited increases in the range of 2- to 3-fold at 3x AA concentrations. NAP showed almost 2-fold increase at 2x-AA with, surprisingly, a slightly lesser increase at 3x-AA. Overall, the chosen amino acid combinations as shown in the precursor matrix (Figure 3a) were thus deemed impactful in increasing the respective intermediate metabolites, leading to the construction of a precursor matrix for the following design study.

## 3.3 | Design study

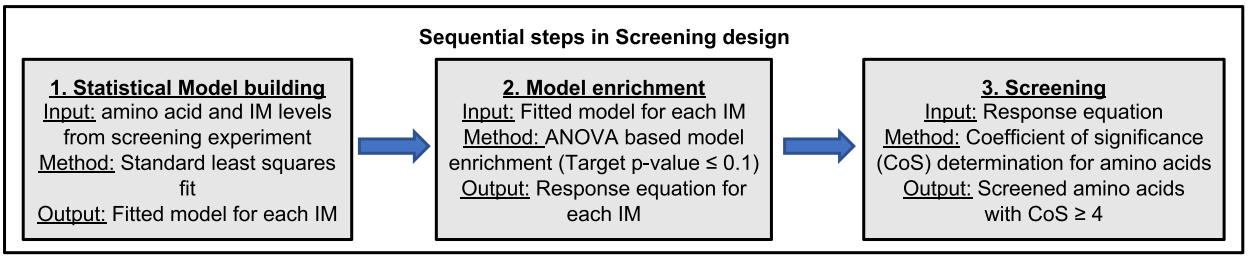
Following the initial evaluation of the amino acid precursors, a Design of Experiments (DOE) approach was implemented in three stages. The first stage involved a screening design to rank the amino acid precursors in order of their contribution toward buildup of the inhibitory metabolites. This would allow us to narrow down the list of amino acids and focus on the most significant contributors. These most significant contributors were then taken forward for a second stage of DOE analysis that utilized a response surface design (RSD) approach to find the potential optimal levels of the most relevant contributors. Finally, stage 3 of the study involved the validation of our optimized AA concentrations from the previous DOE studies with the end goal of evaluating its impact on cell growth and product titers. Figure 1b summarizes the overall methodology followed for the design study to determine the optimal AA concentrations.

### 3.3.1 | Screening design

A two-level screening experiment was performed to identify the amino acids that contributed the most towards accumulating inhibitory metabolites. The precursor matrix with 10 different amino acids shown in Figure 3 was used as input for constructing the screening design. An additional 11th amino acid (Ala) was added as a dummy variable to estimate possible error in the design experiment. Unlike the preliminary study, where the amino acids were increased by two and three times of the basal medium level, this two-level screening design matrix included a high (+1) and a low (−1) value for each amino acid substrate. Since the goal of the screening design was to reduce amino acid concentrations, basal medium levels were designated as the high (+1) level for each amino acid in the design space. For determining the low (−1) level for the amino acids, the consumption profile of each amino acid (Figure 2) was used as input to estimate how much an amino acid can be reduced with minimal impact on growth. The concentration of the dummy variable (11th factor) was kept constant for both cases to help quantify any variability and eliminate any bias in the design. The two levels chosen for each amino acid in this screening design are listed in Figure 4.

The nonessential amino acids were in general tested at a range between 25% and 75% of the nominal level for their low-level value. In choosing the low (−1) levels of the essential amino acids, the maximum reduction was 50% to avoid any detrimental effects on growth due to potential exhaustion in the basal medium. However, leucine and arginine were only reduced by 25% to minimize the

(a)




(b)

| S. No. | Amino Acid | High Level (+1) | Low Level (-1) |
|---|---|---|---|
| 1 | Gln (Q) | 100% | 50% |
| 2 | Ile (I) | 100% | 50% |
| 3 | Asp (D) | 100% | 25% |
| 4 | Asn (N) | 100% | 50% |
| 5 | Leu (L) | 100% | 75% |
| 6 | Arg (R) | 100% | 75% |
| 7 | Trp (W) | 100% | 50% |
| 8 | Pro (P) | 100% | 50% |
| 9 | Ser (S) | 100% | 25% |
| 10 | Lys (K) | 100% | 50% |
| 11 | Ala (A) | 100% | 100% |

| Condition | Q | I | D | N | L | R | W | P | S | K | (A) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PB00 | +1 | +1 | +1 | +1 | +1 | +1 | +1 | +1 | +1 | +1 | +1 |
| PB01 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 |
| PB02 | +1 | -1 | -1 | +1 | +1 | +1 | -1 | -1 | -1 | -1 | +1 |
| PB03 | -1 | -1 | +1 | +1 | +1 | -1 | -1 | -1 | -1 | +1 | -1 |
| PB04 | -1 | +1 | +1 | +1 | -1 | -1 | -1 | -1 | +1 | -1 | -1 |
| PB05 | +1 | +1 | +1 | -1 | -1 | -1 | -1 | +1 | -1 | -1 | -1 |
| PB06 | +1 | +1 | -1 | -1 | -1 | -1 | +1 | -1 | -1 | -1 | -1 |
| PB07 | +1 | -1 | -1 | -1 | -1 | +1 | -1 | -1 | -1 | -1 | +1 |
| PB08 | -1 | -1 | -1 | -1 | +1 | -1 | -1 | -1 | -1 | +1 | +1 |
| PB09 | -1 | -1 | -1 | +1 | -1 | -1 | -1 | -1 | +1 | +1 | +1 |
| PB10 | -1 | -1 | +1 | -1 | -1 | -1 | -1 | +1 | +1 | +1 | -1 |
| PB11 | -1 | +1 | -1 | -1 | -1 | -1 | +1 | +1 | +1 | -1 | -1 |
| PB12 | +1 | -1 | -1 | -1 | -1 | +1 | +1 | +1 | -1 | -1 | +1 |

**FIGURE 4** (a) Sequential steps constructed in screening design to reduce the precursor design space. (b) Screening design matrix with different amino acids. Thirteen conditions were tested with various combinations of 11 amino acids at high (+1) and low (−1) levels.

impact on cell growth, since consumption of both leucine and arginine in batch culture was higher than the other essential amino acids in the matrix.

A matrix of 12 different combinations was constructed using the high (+1) and low (−1) levels of each amino acid in the precursor matrix (Figure 4b). All 11 amino acids were screened using the 12 different combinations, with assignment for the high (+1) and low levels (−1) randomized to ensure that at least half of the 6 amino acids were tested at low levels (−1) in each combination (except for the control). The basal medium was constructed reflecting each of the 12 combinations and CHO cells were cultured in batch mode in duplicates. The basal medium with all amino acids at high (+1) level corresponding to the original concentrations was used as the experimental control (PB00). The cell growth during the batch culture was monitored to identify any combination that resulted in lower peak VCD than control. All the combinations tested in the screening experiment reached similar or slightly higher peak VCD in batch culture (Supporting Information: Figure S2). Inhibitory metabolites were quantified in the supernatant at the end of the batch culture to provide as input into the statistical analysis.

The "Fit model" tool in the program JMP 14 was used to quantitatively determine the design outcome. This statistical modeling approach was implemented to lower the proposed amino acid design space from 10 and therefore reduce the complexity in constructing media and feeds. The input concentrations of amino acids in each of the 12 screening conditions from PB00 to PB12 and the respective metabolite concentrations measured in each condition were statistically fitted using a standard least squares model. The *p* value of each of the resulting six fitted models, one for each inhibitory metabolite, were estimated using ANOVA. All 11 amino acids in the screening design including the dummy variable were considered contributing to the model unless the *p* value of the fitted model was greater than 0.1. In such a case, a sequential enrichment of the least significant factors was conducted by progressively refining the model and removing the amino acids that did not contribute positively to the model significance determined by the model *p* value. This was repeated until the *p* value of the fitted model approached a value closer to or less than 0.1, making the resulting model the best standard least squares fit, and retention of the amino acids contributing to the inhibitory metabolite levels. An overview displaying the best models for each inhibitory metabolite along with the contributing amino acids for each metabolite and the final *p* value is shown in Figure 5.

The fitted models resulted in response equations (Supporting Information: Table S2) that could predict the metabolite concentrations by using concentrations of amino acids in the model as inputs within the concentration ranges used in the design space. The coefficients of each amino acid factor in the response equation helped predict the amino acid factors leading to accumulation of specific inhibitory metabolites. Amino acids with a positive coefficient in the response equation contributed to accumulation of the inhibitory metabolite as their concentration increased while those with a negative coefficient were suggested to lower the metabolite accumulation as their levels increased. For example, TRI exhibited a positive impact from three amino acids including Ile, Lys and Pro and a negative impact from three amino acids including Asp, Ser, and Trp. Therefore, to optimize the amino

| Metabolite | # of significant factors | Significant AAs (p-value<0.1) | P-value (ANOVA) | Design efficiency | Power of the design | R2 value (ANOVA) |
|---|---|---|---|---|---|---|
| ACA | 2 | Lys (0.03)<br>Asp (0.06) | 0.105 | 79.62 | 0.911 | 0.58 |
| HICA | 6 | Asp (0.01)<br>Ile (0.04)<br>Ser (0.05)<br>Gln (0.05)<br>Lys (0.07)<br>Trp (0.09) | 0.05 | 69.8 | 0.866 | 0.97 |
| MSA | 6 | Ser(0.003)<br>Leu(0.01)<br>Lys(0.03)<br>Asn(0.03)<br>Ile(0.04)<br>Trp(0.07) | 0.0163 | 68 | 0.839 | 0.92 |
| CMP | 4 | Pro(0.02)<br>Asp(0.03)<br>Trp(0.04)<br>Arg(0.06) | 0.0872 | 79 | 0.861 | 0.9 |
| NAP | 5 | Ser(0.01)<br>Gln(0.03)<br>Leu(0.04)<br>Ile(0.04)<br>Arg(0.07) | 0.0902 | 64.4 | 0.799 | 0.76 |
| TRI | 2 | Ser (0.01)<br>Lys (0.02) | 0.0625 | 92.3 | 0.942 | 0.79 |

**FIGURE 5** Standard least square fitting of significant factors for each metabolite along with the model efficiency parameters for the fit.

acid levels in the medium and limit accumulation of inhibitory metabolites, the one strategy to improve growth would be to lower the concentrations of the amino acids with the most collective positive coefficients in the response equation.

To achieve this, all the amino acids in the fitted model with positive coefficients were ranked from 1 to N where N is the total number of amino acids in the response equation with a positive coefficient. Amino acids with the highest coefficient value were assigned Rank N and the next highest was given a Rank of N-1 and so on with Rank 1 assigned to the amino acid with the lowest positive coefficient in the response equation. Then, a Coefficient of Significance (CoS) was calculated for each metabolite using the equation below:

$$CoS_{AA} = \Sigma R_{IM}.$$

Where, $CoS_{AA}$ is the coefficient of significance of the amino acid and $R_{IM}$ is the rank of the amino acid in the response equation of the specific inhibitory metabolite (IM). Shown in Supporting Information: Table S3 are the rankings of each amino acid for the response equations of the inhibitory metabolites and also the CoS values, which ranged from 2 to 12. All the amino acids with a CoS ≥ 4 (Ile, Lys, Leu, Arg, and Trp), except Asn due to its complete utilization in CHO-K1 cultures (Figure 2a), were considered for the next level of DOE amino acid optimization using response surface design approaches.

### 3.3.2 | Response surface design

The 5 amino acids, Leu, Ile, Lys, Arg, and Trp, exhibiting the largest impact on the accumulation of inhibitory metabolites, were next evaluated more in depth to specify their predicted "ideal" concentrations in the basal medium. This was done to refine the previous screening design result where only two levels of amino acid concentrations (+1 and −1) were tested to shortlist the AA precursors that are major contributors to the accumulation of inhibitory metabolites. A Box–Behnken (BB) design, which measures the impact of factors on the response variables by generating a data set consisting of different combinations of the amino acid, was employed. More specifically, all the amino acids were tested at three different levels (+1, 0, and −1) in tandem and the measured response variables (inhibitory metabolites concentration at the end of cell culture) were modeled using a 2nd degree polynomial followed by an estimation of the optimal levels of the input parameters using a standard least squares method.

Performing a Box–Behnken DOE of all the 5 key amino acid contributors simultaneously would evaluate 41 unique conditions. To limit the range of conditions tested, the amino acids were divided into two categories based on their roles as more or less significant contributors to the different metabolites based on their CoS. Specifically, Lys and Ile were chosen owing to their highest CoS and Leu was added to the group given it is a BCAA (like Ile) (Liebich & Först, 1984; Mero et al., 2010; Nowaczyk et al., 1998). These three

AAs were grouped together and considered for the first stage Box-Behnken DOE (herein referred to as BB1). This simplification of the design space reduced the conditions for testing to only 13, allowing ample capacity to conduct these experiments in replicate. The optimized levels of AAs levels from the first BB1 statistical design were then taken forward to optimize the levels of Arg and Trp in the basal medium using a second stage Box–Behnken design, BB2.

The cell culture studies for both stages of the Box–Behnken optimization were conducted in CHO-K1 batch cultures. The design matrix for the first step (Stage 1) of this study is shown in Figure 6a. Two additional control conditions, "complete (basal) medium" (with "+1" levels of all AAs) and "negative control" (with "−1" levels of all AAs), were added to the 13-case design matrix to facilitate the comparison of test cases with extreme scenarios. The amino acids being tested for optimization were added back to Medium A− at appropriate concentrations (−1, 0, +1; with specific levels noted in Figure 6b) to reconstitute the basal media for testing. The varying amino acid levels (variable) along with the measured concentrations of the inhibitory metabolites (response) were used as inputs to fit the response as a function of variables. The JMP 14 algorithm yielded critical value solutions for each amino acid corresponding to each inhibitory metabolite. These critical values refer to the value of amino acid at which the derivative of the response (inhibitory metabolite concentration in our case) equation is zero (see Section 2). These critical values for the AA precursors chosen for the BB1 study were tabulated for all the inhibitory metabolites as shown in Supporting Information: Table S5. The results from the 3 concentration levels (+1, 0, and −1) of Leu, Ile and Lys tested for the BB1 test are shown with the predicted "semi-optimal" levels in Figure 6b. Since the results from BB1 study were used as inputs for a second level of optimization in the BB2 study, the criteria for choosing the first stage "semi-optimal" levels of Leu, Ile, and Lys (from the BB1 study) were not kept highly stringent to eliminate any potential detrimental effects due to nutrient deprivation. For example, the desirable value of Leu arising from BB1 DOE optimization for each metabolite appearing within the "−1" and "0" level of concentrations tested for the BB1 study were averaged, giving us 86% of the "+1" value as the first stage "semi-optimal" prediction (Figure 6b). For Ile, a value of 87% (Table 2) was chosen as the first stage "semi-optimal" concentration (Figure 6b) since this represented the lowest critical value (none of the critical values fell within the "−1" and "0" range of concentrations tested). Lastly, for Lys, the second lowest critical value (76% of the original concentration, Figure 6b) was selected as first stage "semi-optimal" level. The lowest critical value of 54% was disregarded because the AA consumption profiles for Medium A (Figure 2) indicated that half of the initial Lys is consumed during the batch culture and thus reducing Lysine concentration to such a low value (54%) could have resulted in complete exhaustion of this AA. Therefore, the criteria for selection of initial optimal concentrations

**TABLE 2** Optimal levels of amino acids derived from the two levels of Box–Behnken optimization study (BB1 and BB2).

| S. No. | Amino acid | Optimal level |
|---|---|---|
| 1 | Leu | 80% |
| 2 | Ile | 87% |
| 3 | Lys | 76% |
| 4 | Trp | 67% |
| 5 | Arg | 75% |

*Note*: These optimal values were used for subsequent implementation study.

(a)

| S. No. | Condition | Leu | Ile | Lys |
|---|---|---|---|---|
| 1 | Complete medium | +1 | +1 | +1 |
| 2 | Negative control | -1 | -1 | -1 |
| 3 | BB01 | -1 | -1 | 0 |
| 4 | BB02 | -1 | 0 | -1 |
| 5 | BB03 | -1 | 0 | +1 |
| 6 | BB04 | -1 | +1 | 0 |
| 7 | BB05 | 0 | -1 | -1 |
| 8 | BB06 | 0 | -1 | +1 |
| 9 | BB07 | 0 | 0 | 0 |
| 10 | BB08 | 0 | +1 | -1 |
| 11 | BB09 | 0 | +1 | +1 |
| 12 | BB10 | +1 | -1 | 0 |
| 13 | BB11 | +1 | 0 | -1 |
| 14 | BB12 | +1 | 0 | +1 |
| 15 | BB13 | +1 | +1 | 0 |

(b)

| S. No. | Amino Acid | High Level (+1) | Medium Level (0) | Low Level (-1) | "Semi-Optimal" Level |
|---|---|---|---|---|---|
| 1 | Leu (L) | 100% | 90% | 75% | 86% |
| 2 | Ile (I) | 100% | 75% | 50% | 87% |
| 3 | Lys (K) | 100% | 75% | 50% | 76% |

**FIGURE 6** (a) Box–Behnken design matrix 1 with different amino acids, 15 conditions were tested with varying levels (+1: high, 0: medium and −1: low) of Leu, Ile, and Lys (B) Concentration levels of Leu, Ile, and Lys tested for Box–Behnken basal medium optimization 1 (BB1) and the "semi-optimal" values chosen for stage 2.

of the AA factors were kept generous to avoid any detrimental effects due to potential nutrient depletion. It should be noted that any method including the Box–Behnken design can exhibit limitations in mapping out the appropriate response surface. An alternative to Box–Behnken in the space of response surface methodology is central composite design which is a traditional fractional factorial design. This approach could also be beneficial in relatively unknown processes (Kazemian et al., 2021). Future studies should consider multiple complementary approaches in determining the ideal concentrations of target amino acids or other substrates that limit IM accumulation.

The first stage "semi-optimal" concentrations of Leu, Ile, and Lys from the BB1 study were then used when reconstituting the culture media Medium A− for the BB2 study. Shown in Figure 7a,b are the design matrix and the three concentration levels of Trp and Arg used for the BB2 optimization. The additional "complete (basal) medium" (with "+1" levels of all AAs) was added to serve as a positive control for this second stage. A statistical approach similar to the BB1 study was followed for determination of optimal levels of Trp and Arg in the basal medium. The "optimal" values of Trp and Arg were then selected based on the lowest critical values (Supporting Information: Table S6; see Materials and Methods) within the range of concentrations tested arising out of the BB2 optimization study. These results suggested that the levels of Trp and Arg in Medium A could be reduced to 67% and 75% of their original levels to lower the accumulation of inhibitory metabolites (Figure 7b). Following completion of this second stage, the optimal values of AAs from the BB1 study were revisited as a final step to finalize the target levels which would subsequently be used in the following validation study. In the case of Leu, the optimal value was retained at its lowest acceptable critical value, 80% of the original concentration, as indicated in Table 2. The optimal levels of Ile and Lys were kept at 87% and 76%, respectively based on their solutions from BB1. Overall, the design concentrations of significant AA substrates in basal Medium A were lowered to a level predicted based on the response surface methodology resulting from their impact on the inhibitory metabolites.

### 3.3.3 | Implementation study

The amino acid levels predicted from this rational medium design were used to formulate a "reduced medium A" from Medium A−. Reduced medium A was then used in a batch cell culture experiment to compare the performance of cells in the reduced medium against the control medium (Medium A, or complete medium A). Clear differences were observed in the growth and protein titers (Figure 8a,b), as the cells cultured in the reduced A medium attained 13% improvement in peak viable cell density and 7% increase in IgG titer. The cells in the reduced medium A grew at the same rate as the complete medium A until Day 3 as indicated in Figure 8c. However, the growth rate of cells started increasing in the reduced medium A condition starting Day 4 and was 7% higher from Day 4 to Day 5. This could be due to the reduced availability of amino acids that are catabolized into dead end pathway intermediates with potential toxic effects on the cellular performance.

Next, we tested the performance of reducing medium components in a fed-batch process (all conditions listed in Supporting Information: Table S7). The control fed-batch (Condition 1) was reproduced as described in Section 2.3. To test the effect of reduced medium, the basal medium of the fed-batch process (complete medium A) was replaced with reduced medium A (Condition 2) with lowered amino acid levels (Figure 9a). CHO cells cultured in the reduced basal medium (condition 2) maintained higher VCD a from Day 5 onward until the final day of culture (15), attaining an 10% higher peak VCD (Figure 9a (i)) and an 8% increase in titer as compared to condition 1 (Figure 9a (ii)).

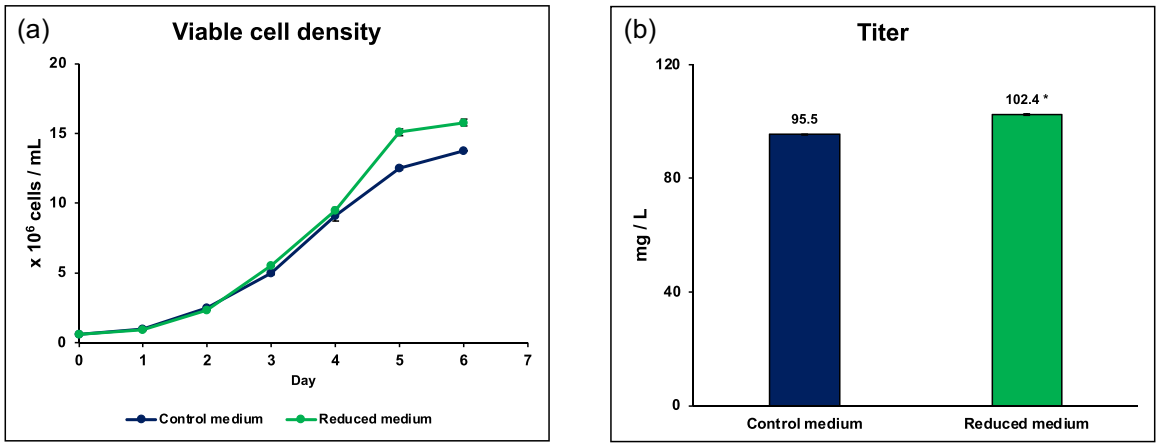
(a)

| S. No. | Condition | Trp | Arg |
|---|---|---|---|
| 1 | Complete medium | +1 | +1 |
| 2 | BB01 | -1 | -1 |
| 3 | BB02 | -1 | 0 |
| 4 | BB03 | -1 | +1 |
| 5 | BB04 | 0 | -1 |
| 6 | BB05 | 0 | 0 |
| 7 | BB06 | 0 | +1 |
| 8 | BB07 | +1 | -1 |
| 9 | BB08 | +1 | 0 |
| 10 | BB09 | +1 | +1 |

(b)

| S. No. | Amino Acid | High Level (+1) | Medium Level (0) | Low Level (-1) | Optimal level |
|---|---|---|---|---|---|
| 1 | Trp (W) | 100% | 75% | 50% | 67% |
| 2 | Arg (R) | 100% | 90% | 75% | 75% |

**FIGURE 7** (a) Box–Behnken design matrix 2 with different amino acids, 10 conditions were tested with varying levels (+1: high, 0: medium and −1: low) of Arg and Trp (b) Concentration levels of Arg and Trp tested for Box–Behnken basal medium optimization 2 (BB2), and their optimal values chosen for next stage.




(c)

| Growth Rate | Control medium | Reduced medium |
|---|---|---|
| Day 3 | 0.727 ± 0.001 $(day)^{-1}$ | 0.721 ± 0.027 $(day)^{-1}$ |
| Day 4 | 0.706 ± 0.006 $(day)^{-1}$ | 0.738 ± 0.014 $(day)^{-1}$ |
| Day 5 | 0.645 ± 0.003 $(day)^{-1}$ | 0.690 ± 0.010 $(day)^{-1}$ |

**FIGURE 8** Cell growth and protein yields from batch implementation study. (a) Viable cell density of CHO cells, (b) IgG titers in cell culture harvest supernatants, and (c) growth rate of CHO cells during exponential phase. Mean ± SD; $n = 2$, $^*p < 0.1$. Statistics by unpaired two-tailed student $t$ test against control (refer Supporting Information: Table S8).

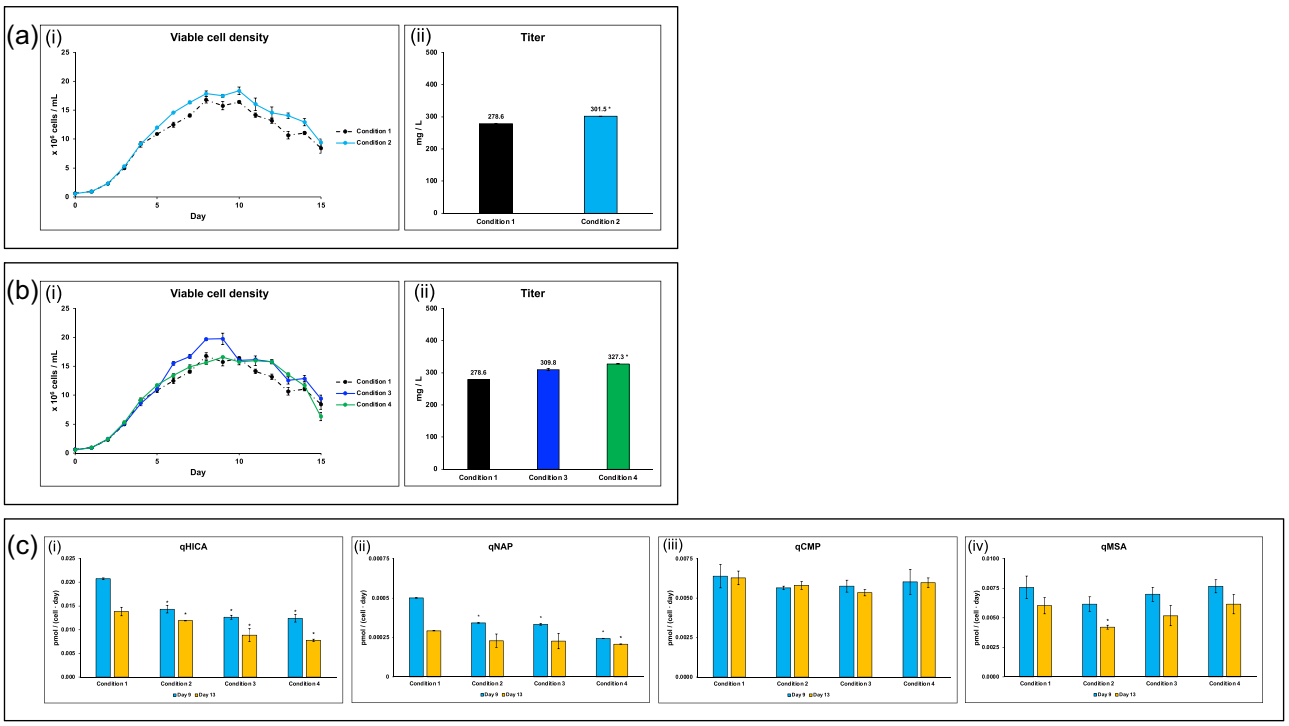



**FIGURE 9** Cell growth characteristics, protein yields, and inhibitory metabolite accumulation in the fed-batch implementation study. (a) (i) Viable cell density of CHO cells, and (ii) end of culture IgG titers in condition 1 and 2. (b) (i) Viable cell density of CHO cells, and (ii) end of culture IgG titers in condition 1, 3, and 4. (c) Specific inhibitory metabolite concentration (per cell accumulation) of (i) HICA, (ii) NAP, (iii) CMP, and (iv) MSA in the spent medium for condition 1, 2, 3, and 4. Spent medium analysis was done for supernatants collected on Day 9 and 13 of the cell culture. Mean ± SD; $n = 2$, $^*p < 0.1$. Statistics by unpaired two-tailed student $t$ test against condition 1 (refer Supporting Information: Table S9). CHO, Chinese hamster ovary.

Furthermore, we wanted to examine if these concepts of reduced amino acids could also be applied to the feed medium (feed B). The concentrations of amino acids in standard feed B were designed to be five times higher than those of basal medium A. We in turn implemented a "reduced feed B" in which the five target amino acids, leucine, isoleucine, lysine, tryptophan, and arginine, were added at 5x levels present in "reduced medium A." Hence, condition 3 tested this effect by replacing feed B with reduced feed B while keeping the same basal medium (complete medium A). We observed that this resulted in an 18% increase in peak VCD (Figure 9b (i)). However, this improvement in cell growth was not sustained, as the VCD for condition 3 dropped to the same level as condition 1 on Day 10, indicating the possibility of nutrient depletion due to a reduction in amino acids in feed B. Nonetheless, condition 3 achieved an 11% titer increase as compared to condition 1 (Figure 9b (ii)). To test the combination effect of reducing the AA levels in both basal and feed media, condition 4 replaced both the complete basal A and feed B with their reduced versions. For this condition, the cells did not attain higher peak VCD but did maintain an extended stationary phase from Day 8 to Day 12 as compared to condition 1. The 17% increase in the IgG titer for condition 4 could potentially reflect this enhanced stability since CHO cells are often more productive during stationary phase (Dean & Reddy, 2013; Rish et al., 2022; Sellick et al., 2015). In fact, lower nutrient availability may have altered cell metabolism, shifting from the growth phase starting on Day 8 and causing the sustained production phase for IgGs in condition 4.

To examine the inhibitory metabolite accumulation in the fed-batch culture conditions tested above, the secreted target metabolite concentrations were measured on Day 9 and Day 13 of the process and plotted as metabolite accumulation on a per cell basis (qMetabolite) as shown in Figure 9c. We observed a reduction in HICA and NAP accumulation in all three fed-batch culture conditions with a 10%–50% decline on Day 9 and Day 13 compared to condition 1 (control). MSA levels were also reduced by 30% and 15% in condition 2 and 3 on Day 13 while they remained relatively unchanged in condition 4. In addition, CMP levels were only slightly reduced between 5% and 15% in conditions 2 and 3, at Day 13, perhaps due to the importance of CMP in many metabolic processes. Overall, the rational medium design approach adopted in this study was successful in achieving reduction in the accumulation of 4 out of 6 inhibitory metabolites under specific media or feed conditions by modulating the amino acid levels. One metabolite, ACA, a TCA cycle related metabolite, previously suggested for tracking the TCA cycle activity, correspondingly remained constant or increased slightly in conditions 2, 3, and 4, perhaps due to enhanced TCA cycle activity. Unlike the other metabolites, TRI was not controlled well with the amino acid optimization strategy likely because the source of TRI also comes from vitamins. Some metabolites will not be controlled well if their sources are not solely due to amino acid catabolic pathways.

Lastly, the observed extension in stationary phase as a result of lowered nutrient availability in condition 4 motivated us to test an alternative feed rate strategy wherein the amount of feed B added to the cultures was reduced from a daily rate of 10% (v/v) to 5% (v/v). Hence, conditions 5 and 6 were tested with the reduced feeding strategy but mimicking the same medium and feed combinations as conditions 2 and 4, respectively. Interestingly, conditions 5 and 6 led to the greatest enhancement in cell growth (Figure 10a). Reducing the feeding volume prolonged the stationary phases of the cultures until Day 12 as opposed to Day 10 in condition 1 (control). Both the conditions (5 and 6) achieved more than 35% and 40% increments in peak VCDs as well as almost 50% enhancement in IgG titers (Figure 10b). Furthermore, these conditions rendered an earlier shift

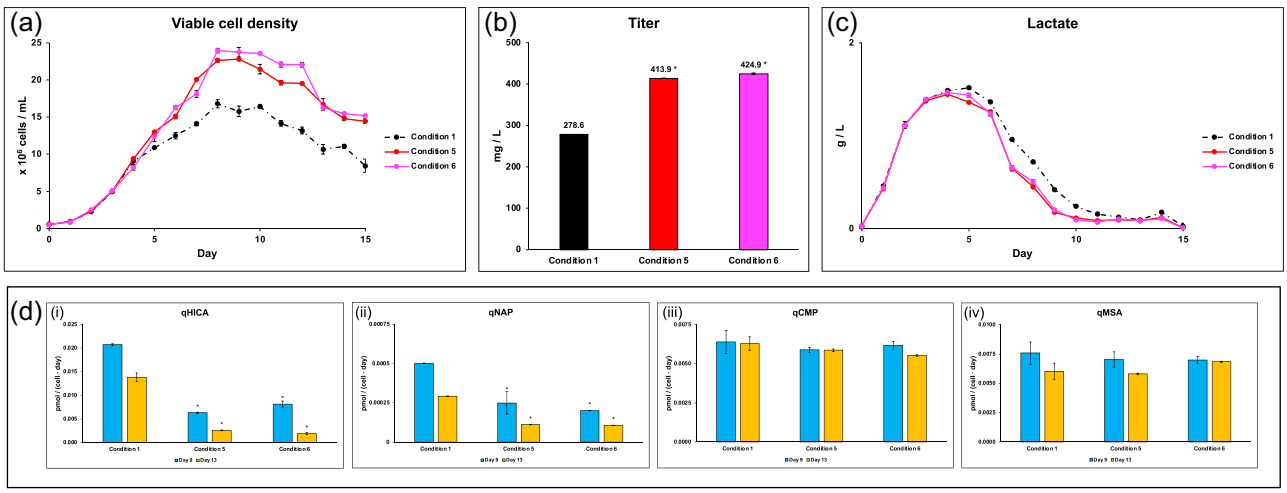



**FIGURE 10** Cell growth characteristics, protein yields, lactate profile and inhibitory metabolite accumulation in the fed-batch implementation study for condition 1, 5, and 6. (a) Viable cell density of CHO cells, [B] end of culture IgG titers, (c) concentration of lactate in the spent medium, and (d) specific inhibitory metabolite concentration (per cell accumulation) of (i) HICA, (ii) NAP, (iii) CMP, and (iv) MSA in the spent medium. Spent medium analysis was done for supernatants collected on Day 9 and 13 of the cell culture. Mean ± SD; $n = 2$, $^*p < 0.1$. Statistics by unpaired two-tailed student $t$ test against condition 1 (refer Supporting Information: Table S9). CHO, Chinese hamster ovary; CMP, cytidine monophosphate; NAP, N-acetyl putrescine.

in lactate metabolism from production to consumption as compared to condition 1 (even as the cells were still growing) to provide evidence of changes in the cell metabolism due to differences in nutrient availability (Figure 10c), which aligns with previous studies associating the lactate metabolic shift to higher TCA cycle activity and higher protein producing fed-batch processes (Mulukutla et al., 2015; Templeton et al., 2013). This improved cell growth and protein titer phenotype also translated to reduction in accumulation of select inhibitory metabolite levels (Figure 10d). Similar to other modified conditions, HICA and NAP had significant ($p < 0.1$) 80% and 60% declines in their accumulation levels on Day 13, respectively.

## 4 | CONCLUDING REMARKS

CHO cells in culture can accumulate problematic inhibitory metabolites. In the current project, we first demonstrated that these inhibitory metabolites can be linked to specific amino acids substrates through metabolic pathway analysis and an accompanying amino acid over-supplementation study. Specifically, we observed that increasing the initial supplemented amount of precursor amino acids up to 200% results in higher accumulation of inhibitory metabolites identified in a previous study. Next, we implemented multilevel statistical DOE analysis to determine the five most critical amino acid targets linked to these inhibitory metabolites in an initial screening study, and secondly to predict how much these amino acids should be reduced to limit inhibitory metabolite accumulation in cultures. We observed that reductions in the starting concentrations of Leu, Ile, Lys, Trp, and Arg were especially impactful on the inhibitory metabolites, HICA, NAP, CMP, and MSA. Finally, we applied these predictions to modulate the levels of the five target amino acids in a DOE-driven, rational modification of culture basal and feed medium. Implementation of these modified basal and feed media with reduced amino acid levels resulted in improved overall cell culture performance along with a reduction in inhibitory metabolites in both batch and fed batch conditions to demonstrate that "less can be better" in media design. Indeed, we saw enhancements in the viable cell densities and, in some cases, extension of the more productive stationary phase with improvements in the overall IgG titers, by reducing initial levels of select amino acids in the basal media and/or feeds by up to 1/3. Thirteen and 7% improvements in peak VCD and IgG titer, respectively, were observed for a batch CHO process. When translated to a fed-batch process, these enhancements varied depending on the combination of basal and feed media suggesting intertwined relationships between nutrient utilization and cell metabolism during different fed-batch phases. Indeed, the inhibitor metabolites HICA and NAP were reduced by up to 50%, MSA by 30%, and CMP by 15% in cultures with modified media. Interestingly, the most significant impact on culture performance was evident when modifications in the basal and fed media were combined with a reduction in the overall feed rate, which lowered the amino acids available to the cells in culture over the extended stationary phase and increased VCD and antibody titers by 40% and 50%, respectively. In this way, the study demonstrates the danger of overfeeding and the value of tuning substrate levels in media formulation based on cellular metabolism to lower problematic by-products while enhancing cellular performance for production of recombinant proteins. Furthermore, the advent of defined reference media formulation such as AMBIC 1.0 and its descendants will serve to create a knowledgebase around how to build a more effective basal and feed media formulation for the entire cell culture community. Studies such as this one will enable researchers and users to better understand the relationships between amino acid and other media components and problematic inhibitory metabolites and metabolism in general that will yield greater insights on how basal and feed media formulation can be adjusted to improve productivities and overall culture performance in the future.

### AUTHOR CONTRIBUTIONS

**Pranay Ladiwala**: Conceptualization; methodology; investigation; formal analysis; validation; writing—original draft; writing—review & editing; visualization. **Venkata Gayatri Dhara**: Conceptualization; methodology; investigation; formal analysis; validation; writing—original draft; writing—review & editing; visualization. **Jackson Jenkins**: Investigation; formal analysis; validation; writing—original draft; writing—review & editing. **Bingyu Kuang**: Investigation; formal analysis; validation. **Duc Hoang**: Investigation; formal analysis; validation. **Seongkyu Yoon**: Conceptualization; methodology; investigation; supervision; project administration; funding acquisition. **Michael J. Betenbaugh**: Conceptualization; methodology; investigation; writing—original draft; writing—review & editing; supervision; project administration; funding acquisition.

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### CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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