Amino acid feeding reduces ammonia production through rearrangement of metabolic fluxes in central carbon metabolism of CHO cells

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Abstract

Ammonia is a toxic byproduct of CHO cell metabolism, which inhibits cell growth, reduces cell viability, alters glycosylation, and decreases recombinant protein productivity. In an attempt to minimize the ammonium accumulation in cell culture media, different amino acids were added individually to the culture medium before the production phase to alleviate the negative effects of ammonium on cell culture performance. Among all the amino acids examined in this study, valine showed the most positive impact on CHO cell culture performance. When the cultured CHO cells were fed with 5 mM valine, EPO titer was increased by 25% compared to the control medium, and ammonium and lactate production were decreased by 23 and 26%, respectively, relative to the control culture. Moreover, the sialic acid content of the EPO protein in valine-fed culture was higher than in the control culture, most likely because of the lower ammonium concentration. Flux balance analysis (FBA) results demonstrated that the citric acid cycle was enriched by valine feeding. The measurement of TCA cycle activity supported this finding. The analysis revealed that there might be a link between promoting tricarboxylic acid (TCA) cycle metabolism in valine-fed culture and reduction in lactate and ammonia accumulation. Furthermore, in valine-fed culture, FBA outcomes showed that alanine was excreted into the medium as the primary mechanism for reducing ammonium concentration. It was predicted that the elevated TCA cycle metabolism was concurrent with an increment in recombinant protein production. Taken together, our data demonstrate that valine addition could be an effective strategy for mitigating the negative impacts of ammonium and enhancing glycoprotein production in both quality and quantity.

Introduction

Chinese hamster ovary (CHO) cells became the preferred mammalian host for industrial production of therapeutic glycoproteins (Kastelic et al. 2019; Walsh 2010) such as monoclonal antibodies (mABs) (Hennicke et al. 2019; Huang and Yoon 2020; Jiang et al. 2019), erythropoietin (EPO) (Ghafuri-Esfahani et al. 2020), interferon (Kochanowski et al. 2008), and coagulation factors (Lalonde and Durocher 2017). During the past decades, different methods have been proposed to improve the volumetric productivity of CHO cells to overcome the challenge of the increasing demand for therapeutic proteins (Bulté et al. 2020; Savizi et al. 2019). One of the major approaches to overproduce biotherapeutic drugs is CHO cell cultivation at the higher level of essential cell culture media elements, particularly glutamine as the main nitrogen source (Sheikholeslami et al. 2014; Xu et al. 2014). However, this approach leads to the accumulation of toxic byproducts, in particular ammonia (Aghamohseni et al. 2014).

Metabolism and the chemical decomposition of glutamine are the primary sources of ammonia buildup in cell culture media (Schneider et al. 1996). The other sources of ammonia in media include the catabolism of some other amino acids such as asparagine (Duarte et al. 2014). High levels of ammonia have adverse effects on CHO cell growth (Chitwood et al. 2021), product titer, and protein glycosylation (Gawlitzek et al. 2000; Savizi et al. 2021). It was observed that the addition of ammonia above 5 mM to the media inhibited CHO cell growth and reduced the volumetric productivity of human erythropoietin (EPO) (Yang and Butler 2000b). In other studies, a decrement in ammonia accumulation resulted in a

doubling of tPA maximum titer (Kim et al. 2013) and enhanced the production of recombinant immunoglobulin (rIgG) (Hong et al. 2010). Furthermore, elevated ammonia concentrations in CHO cell cultures reduced the extent of terminal sialylation of camelid-humanized monoclonal antibody (Aghamohseni et al. 2014) and EPO (Yang and Butler 2000a). Hence, acquiring effective strategies for decreasing ammonia accumulation in a CHO cell culture medium is indispensable.

Various strategies have been developed to reduce the secretion of ammonia by CHO cells. In one approach, researchers have attempted to decrease the production of ammonia via the metabolic engineering of CHO cells. For instance, Ley and coworkers reduced the secretion of toxic byproducts, including ammonia, by reprogramming amino acid metabolism in CHO cells with the CRISPR/Cas9 gene-editing tool (Ley et al. 2019). In another study, two genes related to the urea cycle, which transform ammonia to citrulline, were introduced to CHO cells and resulted in less accumulation of ammonia in media (Park et al. 2000). Media manipulation is the conventional strategy to reduce ammonia production (Ha and Lee 2014; McAtee Pereira et al. 2018). For example, It was shown that maintaining the glutamine concentration at a low level in a fed-batch culture is an effective method for ammonia reduction (Chee Furng Wong et al. 2005). More practically, the substitution of glutamine by less ammoniagenic compounds such as glutamate (Hong et al. 2010), gluten hydrolysate (Kim et al. 2013), or the tricarboxylic acid cycle intermediates (Ha and Lee 2014) led to a decrease in ammonia excretion.

Chen and Harcum illustrated that the addition of amino acids to media is an effective method to mitigate the adverse effects of ammonia on CHO cell cultures (Chen and Harcum 2005). They reported that 20 mM additions of proline, glycine, and threonine could protect CHO cells from the toxic effects of ammonia. However, in another study by Kishishita et al. (Kishishita et al. 2015), although the concentrations of proline, glycine, and threonine in culture media were retained above 20 mM, the negative effects of ammonia were not alleviated. One of the significant differences between the two previous studies is the ammonia addition method to the growth media. Chen and Harcum externally added ammonia to the media prior to inoculation, but the accumulated ammonia in culture media in the study by Kishishita et al. was the result of glutamine and other amino acid metabolism and ammonia produced intracellularly. The difference between cellular formation and the addition of ammonium is the impact on the mitochondrial pH (Fig. 1) (Schneider et al. 1996). Ammonia is mainly produced inside mitochondria in the form of NH_4^+. The mitochondrial membrane is highly impermeable to ammonium, but ammonia can pass from this matrix. This feature would lead to a net flow of ammonia from the mitochondria to the cytoplasm. The ammonia outflow would result in a decrement in the pH of the mitochondria by leaving the protons behind (Fig. 1A). Externally added ammonia would transport to the cytoplasm via the ammonium transporting proteins and then diffuses to the mitochondria in the form of ammonia which leads to an increase of mitochondrial pH by consuming the protons (Fig. 1B). In general, produced ammonium would decrease the pH of mitochondria, while added ammonium would increase it (Martinelle and Häggström 1993). Therefore, Chen and Harcum (Chen and Harcum 2005) study outcomes may not mimic some actual CHO cell cultivations and may not be applicable for some cases of industrial production of glycoproteins.

Figure 1. Schematic representation of how ammonium perturbs the mitochondrial pH (A) ammonium produced by cellular metabolism, (B) externally added ammonium

In the present study, we explore the impact of the feeding of various amino acids on the reduction of intracellularly produced ammonia rather than externally added ammonia to the growth medium, as previously reported in the literature (Chen and Harcum 2005). We propose that feeding amino acids before the stationary phase could decrease the ammonia production rate through the rearrangement of metabolic fluxes in central carbon metabolism. To corroborate this postulation, the nutrient depletion and metabolite accumulation rates were measured during the amino acid feeding and control culture (no amino acid fed) experiments. Then, the experimental data were used with the CHO genome-scale metabolic model (GEM) (Hefzi et al. 2016). This GEM was utilized to analyze the metabolic impact of amino acid addition on ammonia production rate and how the metabolic network is regulated and rewired in response to amino acid feeding. Furthermore, to elucidate the mitigation effect of amino acid addition on the protein sialylation, the sialic acid content of the target glycoprotein was determined.

Materials And Methods

Cell Culture

The rCHO cell line producing human EPO (gifted from Saman Daroye Hashtom, Iran) was used in this study. The CHO cells were cultivated in EX-Cell Advanced (Sigma-Aldrich, St. Louis, MO, USA) and were supplemented with 8 mM L-glutamine (Gibco, Life Technology, USA). CHO cells were cultured in 125 ml Erlenmeyer shake flasks (Corning, Corning, NY) with a working volume of 35 ml. The flasks were agitated at 130 rpm on a shaker which was installed in a CO_2 incubator. The cells were maintained at 37 ^oC, 5% CO_2, and 88% humidity. CHO cells were inoculated at 4×10^5 cells/ml into each flask. Cell viability was specified daily by the Trypan Blue method with Neubauer chamber. Samples for analysis were taken from each flask every 24 h and then was centrifuged at 1100 rpm for 5 min. The supernatant was filtered with a syringe filter (0.22 µm) and saved at -20 °C for further analyses. Cultures were harvested once the cell viability dropped to less than 70%. All cultures were performed in batch mode and were run in duplicates.

EPO Protein Purification

The recombinant EPO secreted into the cell culture medium was purified by reversed-phase chromatography (Yoon et al. 2005). EPO was separated from culture supernatant by loading the samples onto a reversed-phase column (RESOURCE RPC 3 mL, GE Healthcare). The EPO was separated from the samples by utilizing gradient elution: eluent A was composed of 10 mM Tris (pH 7), and eluent B was composed of 10 mM Tris and 80% ethanol (pH 7). The peak related to EPO was observed at a concentration of 65% ethanol, and SDS-PAGE and Western blot assessed the purity of obtained EPO. Then, the buffer of purified EPO protein was exchanged with deionized water through the dialysis process at 4 ^oC.

Determination of glucose, ammonia, lactate, and EPO sialic acid contents

The glucose content of samples was determined by an enzymatic assay kit (Abcam, USA), as per kit instructions. Ammonia concentration was measured based on an ammonia detection kit (Sigma-Aldrich, USA) regarding the instruction manual. According to kit instructions, lactate determination was conducted based on a lactate assay kit (R-Biopharm, Germany). The sialic acid content of purified EPO was determined by utilizing the EnzyChrom sialic acid assay kit (BioAssay Systems, CA), following the manufacturer's protocol.

Amino Acids Analysis

The concentrations of amino acids were measured by HPLC with a reversed-phase column (C18 column, Agilent, USA). Derivatization of amino acids was performed before HPLC analysis using o-phthalaldehyde (OPA). L-α-amino-n-butyric acid was added to each sample as an internal standard. A fluorescence detector detected the derivatives, and the output peaks were identified based on retention time (Walker and Mills 1995). Cysteine and proline concentrations could not be measured with this method. Hence, cysteine and proline assay kits (Abcam, USA) were used to determine the level of these amino acids.

Anti-EPO enzyme-linked immunosorbent assay (ELISA)

The amount of secreted EPO was quantified using the human EPO ELISA kit (Roche, Basel, Switzerland) according to the manufacturer’s instructions. Briefly, supernatants from each sample were added to the anti-EPO precoated microplates. Subsequently, the anti-EPO antibody conjugated to HRP was applied. After washing steps for removing unbound substances, immobilized peroxidase was developed in the complex. Next, the chromogenic substrate (TMB) was loaded to the wells, and then the reaction was stopped with acid. The color intensity, which is proportional to the EPO concentration, was measured spectrophotometrically at 450 nm.

Quantification of intracellular α-ketoglutarate and Citrate synthase activity

The required amount of CHO cells (depending on the test) was collected and were incubated in cold methanol for 5 min. Then, collected samples were washed twice, resuspended in cold PBS, and stored at -80 ^{o}C for further analysis. CHO cells were lysed via sonication and were centrifuged at 10000 g for 10 min (4 ^{o}C). The supernatant was used for the measurement of α-ketoglutarate concentration and citrate synthase activity. The concentration of α-ketoglutarate was measured by a microplate assay kit (Abcam, USA), following the kit's instructions. Citrate synthase was quantified with an enzymatic assay kit (Abcam, USA), as per kit instructions.

Metabolic rates calculation

The specific growth rate (μ) at the exponential phase was determined for two-time points (t_1 and t_2) using the following equation (Equation 1).

$$\mu = \frac{\ln(X_2) - \ln(X_1)}{t_2 - t_1} \quad (1)$$

Where X is the viable cell density. For calculating the consumption and production rates of metabolites based on cell-hour, the integral of viable cell density (IVCD) over two times was defined as cell-hour (ΔCH) and is given in Equation 2 (Reimonn et al. 2016).

$$\Delta CH = \int_{t_1}^{t_2} X dt \approx \frac{X_1}{\mu} \times [\exp(\mu t_2) - \exp(\mu t_1)] \quad (2)$$

The specific uptake or secretion rate (r_c) for any metabolite (C) could be calculated by Equation 3 (Reimonn et al. 2016).

$$r_c = \frac{C_2 - C_1}{\Delta CH} \quad (3)$$

A negative metabolic rate illustrates an uptake rate, and a positive rate describes a secretion rate. In case that any metabolite (C_0) was fed to the culture during the two measurement times (C_1, C_2), equation 3 was converted to the following equation.

$$r_c = \frac{C_2 - (C_1 + C_0)}{\Delta CH} \quad (4)$$

Flux balance analysis using genome-scale models

A genome-scale metabolic model of CHO cells (iCHOv1) (Hefzi et al. 2016) was employed in this study to investigate the metabolism of CHO cells cultivated in batch mode experiments fed with different amino acids. This model can be downloaded from the BIGG database and contains 1766 genes, 4456 metabolites, and 6663 reactions. FBA was applied to determine the intracellular metabolic fluxes through the optimization of a specified objective function by utilizing linear programming. The specific uptake and secretion rates of metabolites including 20 amino acids, glucose, ammonium, and lactate were employed as experimental metabolic constraints. The upper and lower limits of these exchange reaction rates were set to the measured amounts. The specific EPO productivity was also utilized as a constraint, and the optimal flux distribution was acquired by maximizing the biomass reaction flux. We used the default amount from Hefzi et al. (Hefzi et al. 2016) for the other flux boundaries, such as oxygen consumption rate. Alternate optimal solutions were assessed by LAMOS algorithm (Motamedian and Naeimpoor 2018) in the present study. LAMOS was utilized to specify the flux ranges of the desired reactions by determining the minimum and maximum values of those fluxes. To avoid the degeneracy in the FBA problems, the Manhattan norm of the fluxes was minimized while the specific growth rate was

set to its optimal value. FBA in this study was performed by the Constraint-Based Reconstruction and Analysis (COBRA) toolbox v3.0 (Heirendt et al. 2019).

Results

Effect of amino acid addition on cell growth, ammonia, lactate, and EPO production

To specify the effects of amino acid feeding on CHO cell culture performance, different amino acids (leucine, valine, lysine, serine, glycine, proline, and threonine) were fed to the culture when the ammonium concentration in media reached an elevated level. Serine (Horvat et al. 2020), glycine, proline, and threonine (Chen and Harcum 2005) were selected because they were shown in different studies to mitigate the negative effects of ammonium. Leucine, valine, and lysine were chosen based on the following features: (1) they only release their amine by transamination reactions in CHO cells and add no more ammonia to the cell according to the KEGG pathway database (Kanehisa and Goto 2000); (2) they can supply anaplerotic substrates to support TCA cycle anaplerosis. To find the impact of amino acids on CHO cell growth and secretion of metabolites and recombinant EPO, cells were cultured in shake flasks, and each selected amino acid as an individual feed was added to the culture on day 4 when ammonium concentration became higher than 5 mM and before cells entered the stationary phase. We used 5 mM concentration of each amino acid for two reasons: first, the addition of high levels of amino acids would inhibit growth (Pereira et al. 2018); second, we wanted to compare our results with the study conducted by Zhang et al. (Zhang et al. 2020). We also checked and figured out that the concentration of selected amino acids (except proline) did not deplete in the control culture (no amino acid fed) by the end of the batch.

First, we elucidate the profiles of viable cell density (VCD) (Figure 2A). The VCD of all instances peaked on day 5. Amino acid feeding had no discernible effect on maximum viable cell concentration (MVCC) in most cases, and it is almost equal to 15.5×10^6 cell/ml, except for serine and glycine additions. Serine feeding decreased the cell growth rate and MVCC to levels below the control. Glycine also inhibited cell growth but was much more severe than serine (data not shown). Viability in all experiments was higher than 96% until day 5, and it dropped sharply to lower than 80% on day 6 except for the culture fed with valine (87% on day 6). Among added amino acids, valine had the highest EPO titer of 157.5 µg/ml (Figure 2B), which was approximately 25% higher than that in the control culture. The EPO concentration in the lysine and leucine conditions was slightly higher than in the control case, but the level of EPO protein in the threonine and proline conditions was marginally lower than in the control culture (Figure 2B). The lowest EPO titer among all amino acids is related to the serine condition equal to 98 µg/ml (28% lower relative to the control). Despite the differences in the maximum EPO protein concentrations, the q_{EPO} was nearly similar for all amino acids except valine which was almost 1.19 times higher than that with the control experiment.

One important metabolic effect of amino acid feeding is on ammonium metabolism. As illustrated in Figure 2C, the valine addition resulted in a 23% reduction in ammonium production by CHO cells

compared to the control medium. Although it is not so significant, feeding of lysine and leucine led to a 9 and 7% decrease in final ammonium concentration, respectively. The ammonium levels in proline- and threonine-fed cultures were approximately the same as the ammonium level in the control culture. As observed in serine-fed culture, the final ammonium level reached 9.5 mM, which was remarkably higher than that with the control medium.

Another significant impact of amino acid feeding was on lactate with 26, 8, and 6% reduction in final lactate concentrations in valine-, lysine-, and leucine-fed cultures, respectively (Figure 2D). The lactate level in proline-fed culture was almost identical to the lactate extent in the control culture, but feeding threonine caused a slight increase in the lactate concentration. Serine addition elicited a more dramatic 36% increase in lactate production compared to the control medium. The lactate was only consumed in the culture fed with valine on day 7.

Figure 2. CHO cell culture profiles of (A) viable cell density, (B) EPO protein concentration, (C) ammonium concentration, (D) lactate concentration. All experiments were run in duplicates.

Specific consumption and production rates of amino acids

According to our results in the previous section, we found that valine has the most positive effect on cell culture and EPO production. Hence, to further understand the impacts of valine feeding on cell culture performance, specific consumption and production rates of amino acids for valine-fed and control culture during the exponential growth phase (day 0 to day 5) have been assessed (Figure 3). Since the valine-fed culture produced more EPO protein than the control medium, it could be expected that valine feeding would result in higher specific consumption rates of amino acids. However, the specific uptake rates for asparagine, aspartate, cysteine, serine, tyrosine, and glutamine were higher in the control culture. The specific consumption rate of valine was higher in valine-fed culture since the specific amino acid uptake rates depend on the extent of corresponding amino acids in the medium (Fan et al. 2015; Geoghegan et al. 2018). Alanine, glutamate, and glycine were secreted in all experiments by CHO cells, and their production rates were higher in the culture fed by valine. Among all the produced amino acids, alanine production was increased remarkably in valine-fed culture compared to the control medium.

Figure 3. Specific amino acids production (+) and consumption (-) rates during the exponential growth phase. Valine-fed culture (dark blue bar), control culture (light blue bar). Measured values are the means of two independent experiments. Consumption and production rates measured during the exponential growth phase (day 0 and day 5)

Intracellular flux quantification by the genome-scale model of CHO cell

To explore the intracellular pathway variations associated with valine feeding, steady-state metabolic fluxes were calculated by applying the genome-scale model. The predicted cell growth rates (control and valine-fed) were similar to the experimental measurements during the exponential growth phase. This consistency between experimental data and model simulation has been observed by other

researchers (Hefzi et al. 2016; Huang et al. 2020) and demonstrates that the genome-scale model used in this study can predict phenotypes. The intracellular flux alterations are shown in Figure 4 by illustrating the glycolysis and TCA cycle flux ratios (ratios of metabolic fluxes under the valine-fed condition to the same fluxes under the control condition). LAMOS confirmed that for most of the fluxes of interest, the minimum and maximum amounts were the same and equaled the values predicted by FBA (Table S1 and S2). However, some of the reported fluxes had different minimum and maximum values. For the latter fluxes, flux ranges of the desired reactions for the control did not overlap with valine-fed. LAMOS was used instead of flux variability analysis (FVA) in this study since the FVA results (data not shown) showed that some reactions including TCA cycle reactions had an infinite flux range. It was demonstrated since LAMOS could exclude the futile cycles from the optimal solutions, the results of uncertainty analysis by this algorithm would contain no unconstrained reactions (intracellular reaction with infinite flux range) (Motamedian and Naeimpoor 2018).

As shown in Figure 4, although the specific glucose uptake rate was decreased in valine-fed culture, some fluxes of glycolytic reactions were increased (fluxes between glucose 6-phosphate (G6P) to 3-phospho-D-glycerate (3PG)) because less flux was diverted into the pentose phosphate pathway (PPP). Since more of 3PG converted to serine, less of this compound transformed to pyruvate and entered the TCA cycle under the valine-fed condition. The uptake rate of valine was increased in the valine-fed batch compared to the control medium, and most of this component converted to the succinyl-CoA and entered the TCA cycle. Figure 4 indicates that TCA metabolism was enriched as a result of the valine added to the culture. With increasing the concentration of α-ketoglutarate, glutamate dehydrogenase was working in the direction of glutamate (ammonium consumption path). Then glutamate transferred its ammonia to pyruvate, resulting in an increase in the flux from pyruvate to alanine and a reduction in the flux from pyruvate to lactate. Under the valine-fed condition, less α-ketoglutarate transformed to succinyl-CoA and resulted in a higher level of glutamate. A higher concentration of glutamate may be the reason for the lower uptake rate of glutamine (McAtee Pereira et al. 2018).

Figure 4. The glycolysis, TCA cycle, and ammonium removal mechanism flux ratios (ratios of metabolic fluxes under the valine-fed condition to the same fluxes under the control condition) were calculated by the FBA method.

Analysis of TCA cycle activity

The TCA cycle activity was analyzed by measuring the concentration of α-ketoglutarate (Figure 5A) (Huang et al. 2020). As shown in Figure 5A, the relative concentration of this TCA intermediate is similar between the valine-fed and control culture up to day 4. As the experiments carried on and passed day 4, valine-fed cultures illustrated a substantial increase in α-ketoglutarate. The concentration of this metabolite was increased in valine-fed culture is likely because TCA cycle activity was enhanced as a result of the addition of valine to the culture, which supports the outcomes of the previous section (FBA results). In addition, citrate synthase activity (a biomarker for mitochondrial content) (Larsen et al. 2012)

was observed to be elevated in the valine-fed culture after day 4 (Figure 5B), which further demonstrates a higher activity of mitochondria in the valine-fed culture.

Figure 5. Normalized α-ketoglutarate concentration (A) and normalized citrate synthase activity (B) for valine-fed (blue square) and control culture (orange circle). All experiments were run in duplicates. The concentration or the activity at day 0 in the control culture was set as 100 % and other data points were normalized to that point.

Effect of amino acid addition on sialic acid content of EPO

Different studies showed that the degree of sialylation is a crucial feature of EPO protein since there is a close connection between the sialic acid content of EPO and its biological activity (Fukuda et al. 1989; Morimoto et al. 1996). Hence the sialic acid content of recombinant EPO (moles of sialic acid/mol of EPO) was measured on days 5 and 6 (Figure 6). The sialic acid content of EPO was higher in valine-fed culture than in the control medium on both days. The sialic acid content of EPO protein was decreased with culture time for both valine-fed and control culture, but more slowly with the culture fed with valine. As shown in Figure 6, cultivation at valine-fed condition exerted a positive effect on EPO sialylation, and the product sialylation was increased by 23% and 44% on days 5 and 6, respectively.

Figure 6. The sialic acid content of EPO protein on days 5 and 6. Valine-fed culture (black bar), control culture (gray bar).

Discussion

The main goal of this study was to specify if amino acid feeding could protect CHO cell growth and protein production in the existence of elevated ammonia. Some amino acids such as proline and threonine could not alleviate the negative impacts of ammonium, and even in some cases (serine- and glycine-fed), they had adverse effects on cell growth. Leucine and lysine had slight positive impacts on CHO cell performance. Among all tested amino acids, valine had the most significant positive effect on lactate and specifically ammonium production. Yang and Butler (Yang and Butler 2000b) reported ammonium would inhibit CHO cell growth even at low concentrations (less than 5 mM). In another study, Lao and Toth (Lao and Toth 1997) observed that the presence of lactate in the medium would inhibit CHO cell growth by 25%. It was also reported that high ammonium levels would decrease tissue-type plasminogen activator (t-PA) production in CHO cells (Hansen and Emborg 1994). In the present study, feeding valine improved EPO protein productivity and cell viability by reduction of ammonium and lactate accumulation and mitigating their negative impacts, which is in good agreement with the previously mentioned studies. Also, our results about the effect of feeding valine on suppressing ammonium and lactate production are in good agreement with experimental measurements reported by Hiller et al. (Hiller et al. 1994) for hybridoma cells. They illustrated that feeding valine, leucine, isoleucine, and lysine resulted in a drop in ammonium and lactate concentration and improved cell growth in hybridoma cell culture. They showed that valine and isoleucine (together as a feed) addition had the best positive impact on ammonium level, and the specific production of ammonium was decreased by 35%. In another study

(Schmidt et al. 2020), it was shown that the CHO cell culture with the highest valine concentration produced less ammonium that is consistent with our findings.

During the last 2 days of cultivation, productivity enhancement coincided with the lactate profile switched from production to utilization in the valine-fed culture, while this switch did not happen in other cultures (Fig. 2D). Different studies have indicated that protein production is improved in cell cultures that undergo lactate metabolic shift (production to use) during the stationary phase (Dean and Reddy 2013; Hartley et al. 2018). Although several researchers attempted to elucidate the lactate metabolism shift, the mechanism underlying how lactate switch could enhance recombinant protein production remains to be entirely understood (Martínez et al. 2013; Zagari et al. 2013). Lactate consumption results in valine-fed culture are consistent with previous reports (Le et al. 2012). Our findings also displayed the positive relevance between lactate metabolism shift with EPO productivity. Also, it should be considered that lactate consumption would reduce its concentration and hence mitigates its adverse impact on cell viability (Seth et al. 2006).

In the current study, the MVCC was decreased in the serine-fed medium, and the growth was almost inhibited by the addition of glycine to cell culture, which may be due to the increased level of ammonium in cell culture (Yang and Butler 2000b). We observed the ammonium production was increased in glycine- (data not shown) and serine-fed culture relative to the control medium. McAtee and coworkers (McAtee Pereira et al. 2018) stated that decreasing media concentrations of serine had a significant impact on ammonium production. They stated that serine should be bounded to biologically required extents, and the transformation of serine to pyruvate can be another source of ammonium in CHO cell cultures. Moreover, as observed previously (Duarte et al. 2014), the regulation of serine levels to match cellular metabolic requirements has been illustrated to minimize ammonium accumulation in media effectively. Wahr et al. (Wahrheit et al. 2014) reported serine can be served as an ammonium sink; thus, its supply should be limited to enhance ammonium removal. In another study (Xing et al. 2011), it was shown that glycine production could be assumed as an alternative ammonium sink for CHO cell culture. So, glycine supply from the feed should be reduced to enhance ammonium elimination from the medium. It can be concluded the addition of serine and glycine to the culture probably inhibited the mechanisms behind ammonium removal by these two amino acids. Overall, since this study aims to mitigate the detrimental effects of ammonium through amino acid addition and only valine was effective in suppressing ammonium production, we chose valine as a proper amino acid for further analysis.

In valine-fed culture, the ammonium production was decreased concomitant with the significant increase of alanine excretion (Fig. 3). Alanine is presumed to be secreted into the cell culture as a mechanism to decrease ammonium toxicity because it acts as a nitrogen sink (Synoground et al. 2021; Wahrheit et al. 2014). Our results would confirm this hypothesis that ammonia stress can be compensated through an increased alanine secretion. Glycine and glutamate production rates were raised in the culture fed with valine as well (Fig. 3). As discussed before, glycine can be utilized as a nitrogen sink in CHO cells and is intracellularly synthesized from serine (Narkewicz et al. 1996). Since serine uptake rate was decreased and glycine excretion rate was increased simultaneously, it could be concluded more serine (nitrogen

sink) was produced intracellularly (Fig. 4), which is correlated with a decrease in serine consumption rate. Glutamate synthesis from α-ketoglutarate needs ammonium, and an increase of glutamate production in valine-fed culture could decrease ammonium accumulation in the medium as well. It was shown in different studies that the substitution of glutamine by glutamate would reduce the accumulation of ammonium (Altamirano et al. 2001; Hong et al. 2010). Our results illustrate that the glutamine consumption rate was reduced in the culture with higher glutamate concentration and resulted in a lower ammonium concentration (valine-fed condition). This trend confirms the concept that less ammonium would be produced in cultures that glutamine is replaced by glutamate. The measured uptake rate of aspartate was reduced in the valine-fed medium. This result correlates with the observations reported by Wahrheit and coworkers (Wahrheit et al. 2014), which have shown that the aspartate uptake rate was decreased in ammonia-stressed conditions. They observed that the reduction in aspartate uptake was along with more glycine and glutamate excretion to the medium. This data indicates that glutamate used oxaloacetate to produce aspartate and α-ketoglutarate through the transamination pathways.

Flux balance analysis results (Fig. 4) reveal that although less glycolytic-derived pyruvate entered the TCA cycle, citric acid cycle fluxes were increased in valine-fed culture. The fact that TCA metabolites were enriched is due to the increment in succinyl-CoA content, which is the end product of valine degradation. Valine can serve as an energy source and enrich the TCA cycle by forming energy-rich intermediates (Long et al. 2019; Mattick et al. 2013; Neinast et al. 2019). Different researchers confirmed that the improvement of mitochondrial function would enhance CHO cell growth, viability, and productivity (Dean and Reddy 2013; Sellick et al. 2015; Templeton et al. 2013). The results obtained in the current study would confirm this postulation that boosting TCA cycle metabolism can increase specific recombinant protein production and decrease lactate accumulation (Templeton et al. 2013). Overall, there is a metabolic relationship between valine uptake rate and TCA cycle promotion by providing TCA cycle intermediates via valine catabolism. As we observed in this study, the α-ketoglutarate concentration (Fig. 5A) and citrate synthase activity (Fig. 5B) were increased in valine-fed culture, which illustrates a higher activity of the TCA cycle. Likewise, Huang et al. (Huang et al. 2020) reported that the concentrations of TCA intermediates were increased substantially by an increment in Branched Chain Amino Acids (BCAAs) consumption rates. They showed a higher amount of valine and leucine in the feed could enhance specific IgG productivity and reduce lactate production. They did not report the ammonium concentration in their study. In another research by Zhang et al. (Zhang et al. 2020), succinic acid, the product of succinyl-CoA (the end product of valine metabolism), was fed to the CHO cell culture in the late stage of cultivation. They observed antibody titer was increased by 24%, and the final ammonium level was reduced by 57% in succinic acid-fed culture. Although we used 5 mM valine and they used 5 mM succinate, their ammonium reduction was higher than what we observed in our study. Even though their cultivation process is different from our experiment, one possible reason is that they showed the majority of the succinic acid fed was utilized directly in the citric acid cycle, but in our study, according to FBA results, all of the valine did not convert to the succinyl-CoA and entered the TCA cycle.

Figure 4 illustrated that ammonium reacted with α-ketoglutarate and formed glutamate. Then, glutamate transferred its ammonium to pyruvate, and alanine was produced and secreted to the culture. This is one

of the mechanisms behind the ammonium removal from the cell, which removed the intracellular ammonium according to our FBA outcomes. There are other mechanisms, including more glycine secretion, that would remove ammonium from the cytoplasm. In valine-fed culture, since the increment in the alanine exchange rate (34%) was higher than other ammonium sinks, we concluded that alanine excretion is the primary ammonium removal mechanism. Valine feeding assisted the main ammonium removal mechanism by supplying more α-ketoglutarate through conversion to succinyl-CoA and enriching the TCA cycle intermediates, including α-ketoglutarate. The fact that the α-ketoglutarate concentration (Fig. 5A) was increased in valine-fed culture supports this result. The ammonium detoxification behavior observed in the current study has been reported for skeletal muscle cells (Hayashi et al. 1981). It was illustrated that enhanced rates of BCAAs metabolism would improve ammonium detoxification in skeletal muscle cells and reduce blood ammonium levels (Dam et al. 2018; Holecek 2015).

The sialylation degree of EPO was higher in valine-fed culture relative to the control culture (Fig. 6). Previous work has demonstrated that ammonium concentration in culture contributed to maintaining the sialic acid content of EPO protein (Yang and Butler 2000a). They reported that an increase in ammonium level would be concomitant with a reduction in the sialylation of glycans of EPO, which are analogous to our data. Ha et al. (Ha and Lee 2014) showed that reduced ammonium accumulation had a positive impact on the activity and expression level of α-2,3-sialyltransferase and had a negative impact on extracellular sialidase. Consequently, low ammonium concentration would create a beneficial impact on the product sialic acid content.

In conclusion, it was demonstrated that the addition of 5 mM valine before the stationary phase reduced ammonium accumulation and improved lactate consumption, leading to increased EPO productivity, cell viability, and sialic acid content. Thus, valine addition could be an effective method for mitigating the adverse effects of ammonium and improving glycoprotein quantity and quality. In valine-fed culture, alanine was secreted into the cell culture as the primary mechanism to reduce ammonium accumulation and acted as a nitrogen sink. FBA calculations demonstrated that some central metabolic pathways, including the TCA cycle, were affected entirely by the valine feeding, confirming that amino acid feeding could effectively manipulate important bioenergetic fluxes. We observed promoting TCA cycle metabolism may be a possible method to increase specific recombinant protein production and reduce lactate and ammonia accumulation. Understanding which metabolic pathways are altered by amino acid addition can provide vital information for the formulation of industrial cell culture media

Declarations

Compliance with Ethical Standards

Funding Information

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Author contributions

Iman Shahidi Pour Savizi: Carried out the experiments; Formal analysis; Software; Writing-original draft; Writing-review & editing. Nader Maghsoudi: Provided cells and material; Visualization. Ehsan Motamedian: Software; Supervision; Visualization. Nathan E. Lewis: Supervision; Visualization. SeyedAbbas Shojaosadati: Funding acquisition; Supervision; Validation.

Conflict of interest

The authors declare that they have no conflicts of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Data availability statement

Data will be made available on reasonable request.

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Figures

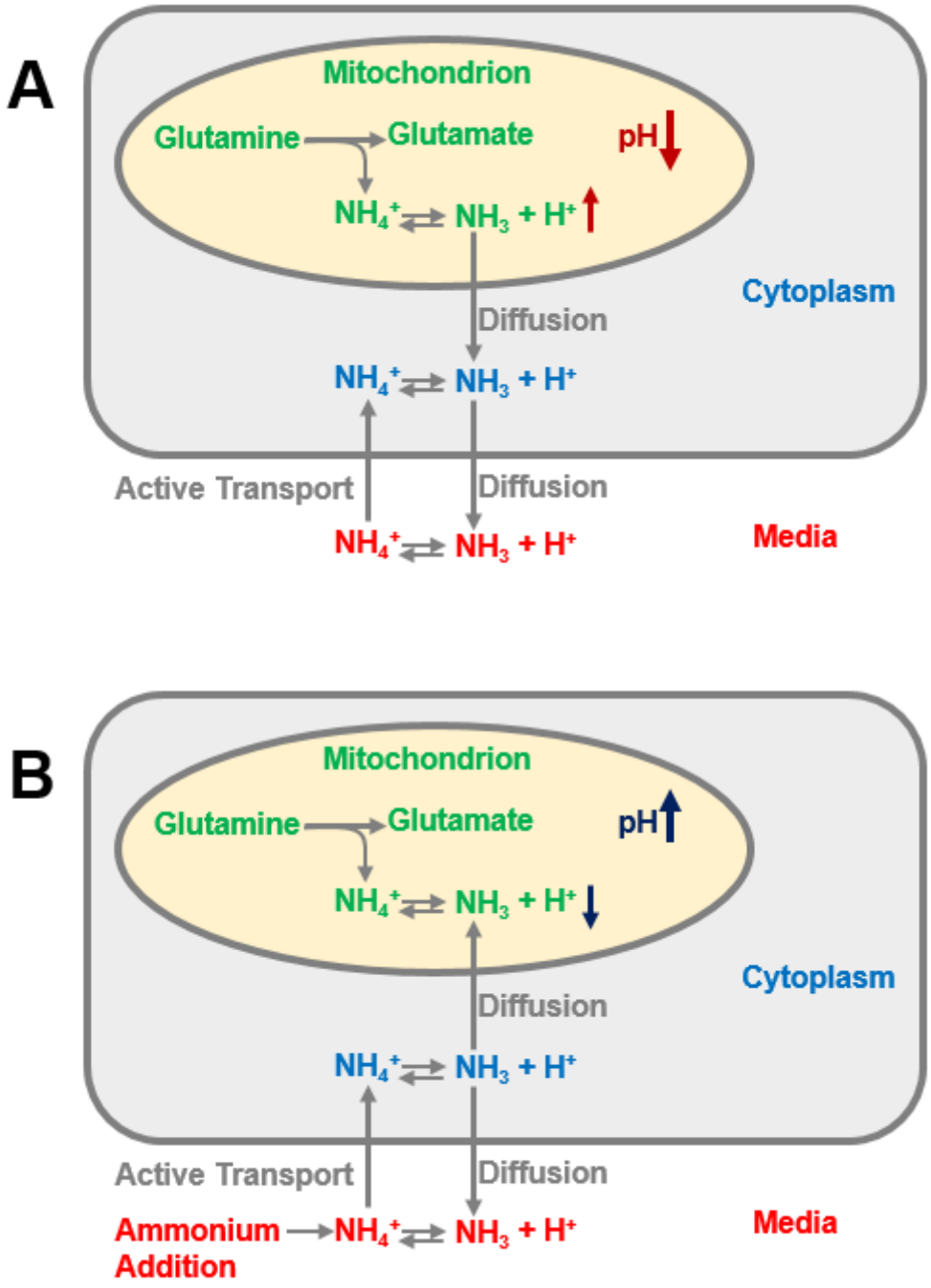

Figure 1

Schematic representation of how ammonium perturbs the mitochondrial pH (A) ammonium produced by cellular metabolism, (B) externally added ammonium

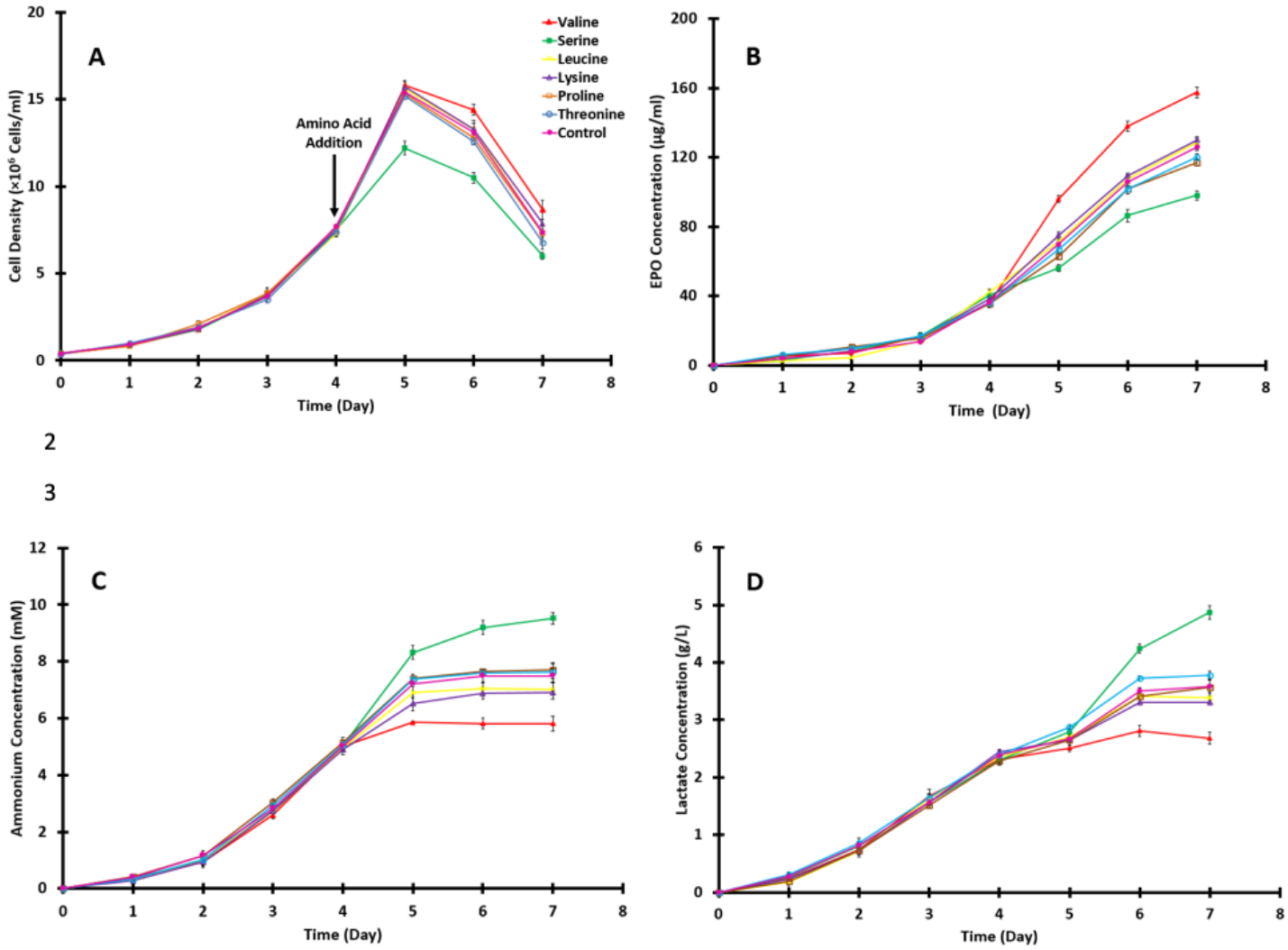

Figure 2

CHO cell culture profiles of (A) viable cell density, (B) EPO protein concentration, (C) ammonium concentration, (D) lactate concentration. All experiments were run in duplicates.

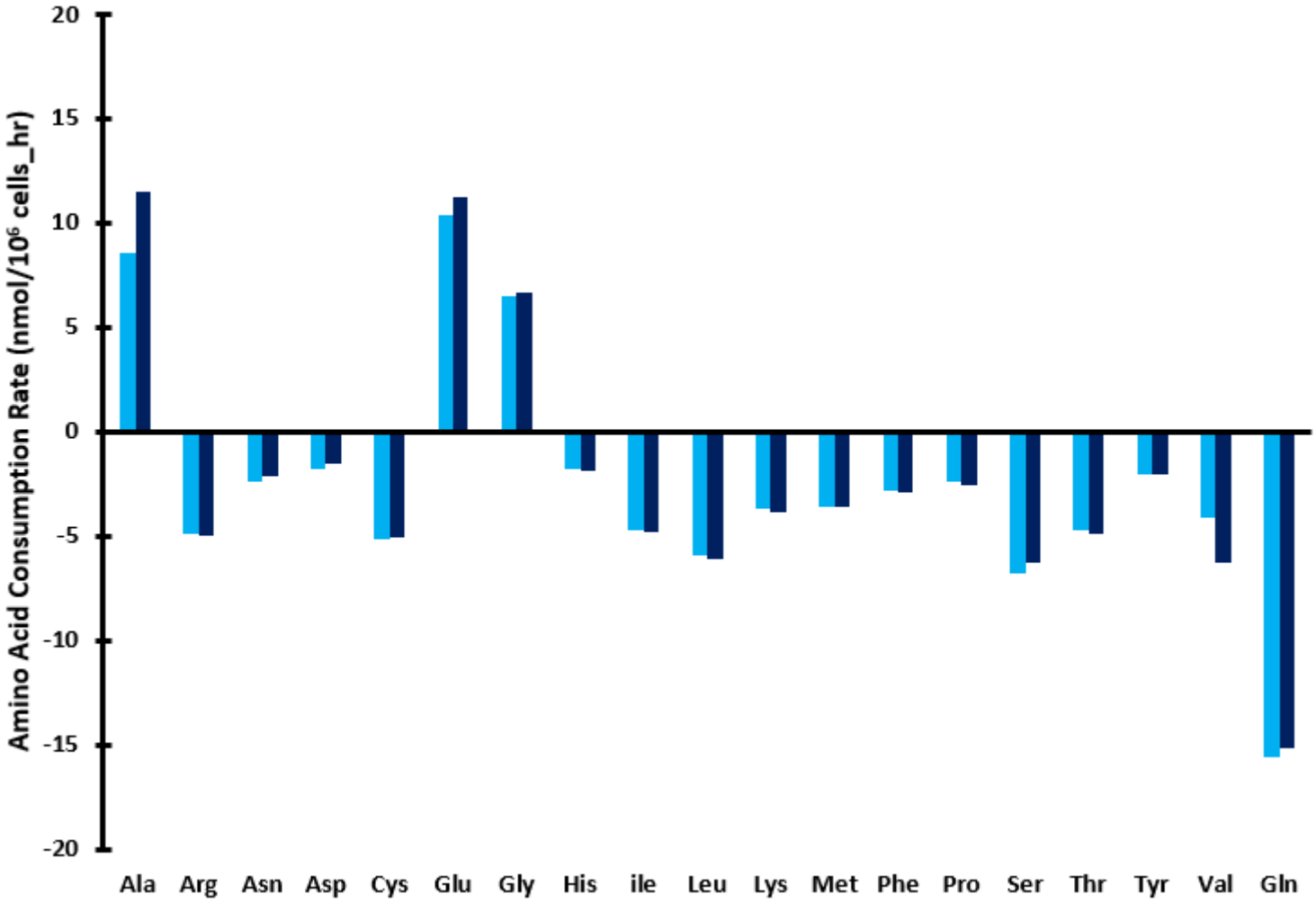

Figure 3

Specific amino acids production (+) and consumption (-) rates during the exponential growth phase. Valine-fed culture (dark blue bar), control culture (light blue bar). Measured values are the means of two independent experiments. Consumption and production rates measured during the exponential growth phase (day 0 and day 5)

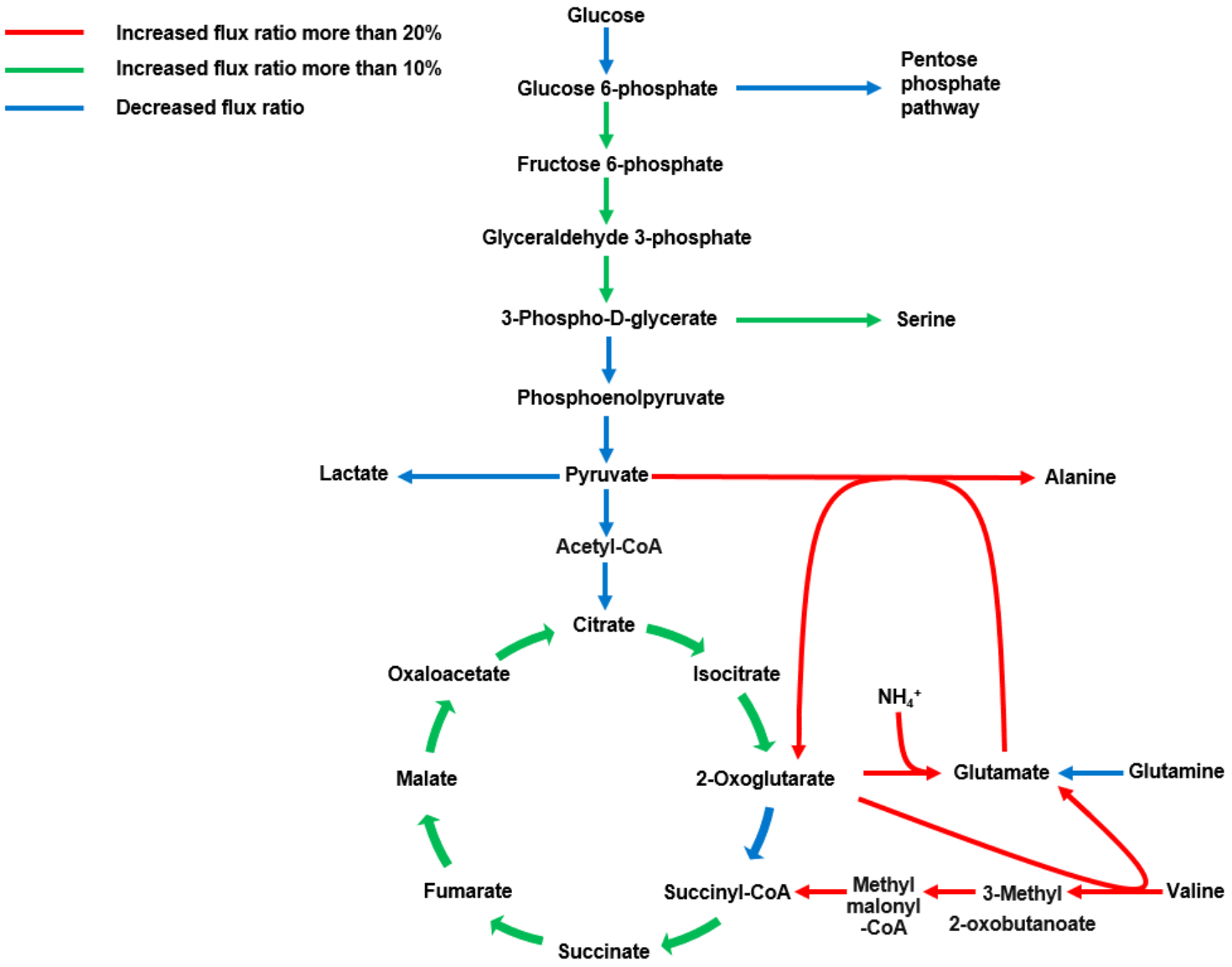

Figure 4

The glycolysis, TCA cycle, and ammonium removal mechanism flux ratios (ratios of metabolic fluxes under the valine-fed condition to the same fluxes under the control condition) were calculated by the FBA method.

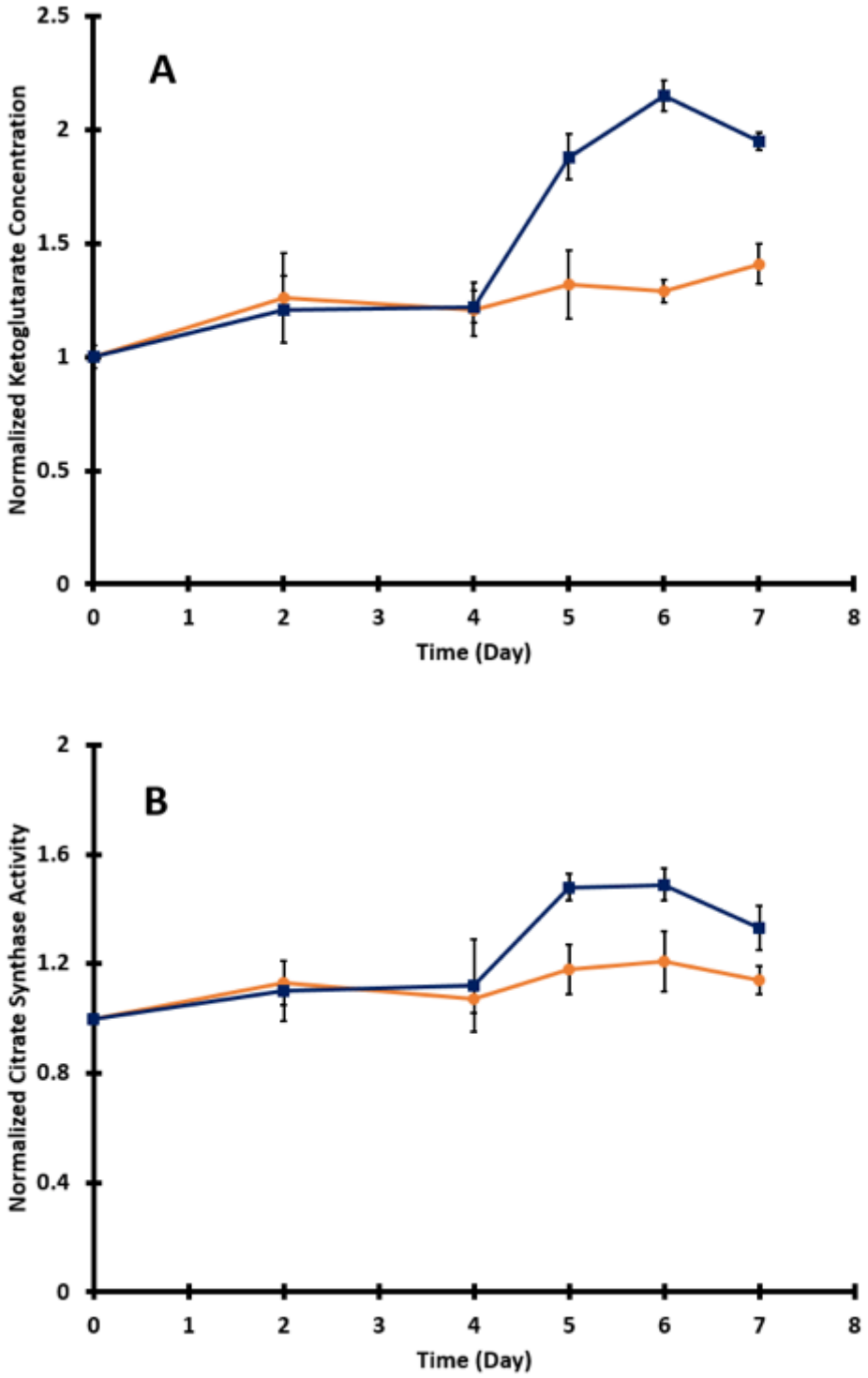

Figure 5

Normalized α-ketoglutarate concentration (A) and normalized citrate synthase activity (B) for valine-fed (blue square) and control culture (orange circle). All experiments were run in duplicates. The concentration or the activity at day 0 in the control culture was set as 100 % and other data points were normalized to that point.

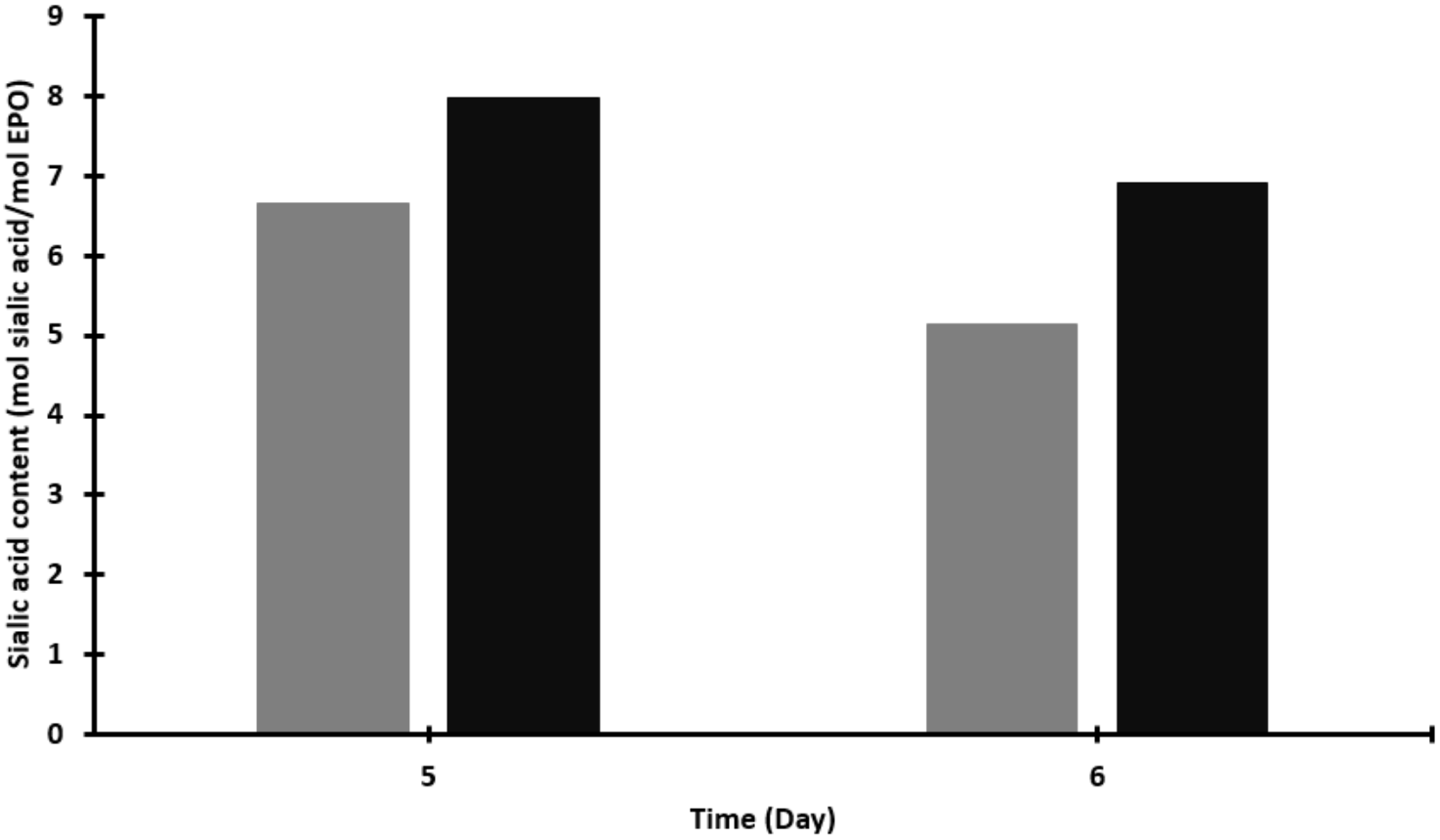

Figure 6

The sialic acid content of EPO protein on days 5 and 6. Valine-fed culture (black bar), control culture (gray bar).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupportingInformation.docx