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Metabolic engineering of *Bcat1*, *Adh5* and *Hahdb* towards controlling metabolic inhibitors and improving performance in CHO cell-cultures

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ABSTRACT

CHO cells are known to secrete growth inhibitory metabolites during growth and production phases, which hampers cellular performance and negatively impacts final productivity and product quality attributes. Previous studies have identified different metabolic by-products derived from CHO metabolism and demonstrated their negative impacts on growth and titer productivity. This work presents a control strategy that incorporated genetic engineering and inhibitory metabolites pathway analysis to regulate cellular metabolism. In this study, three different metabolic genes involving the metabolism of branched-chain amino acids (*Adh5*, *Bcat1* and *Hadhb*) were cloned from CHO reversely synthesized cDNA to study for gene functionality towards regulating cellular metabolism. Identified metabolic genes were individually engineered into expression vector and transfected to cells at plasmid concentration of 40 μg/μL. For all engineered conditions, the peak VCD profile of cells on Day 4 realized a 18–20% increase with cumulative VCD profile realizing 16–19% increase in terms of total viable cells collected on harvest day. Increase in IgG1 titer production and also more complex glycosylation profiles formation were also observed from all engineered subclones. This study hereby successfully demonstrated rewiring cellular metabolism through up-regulation of key metabolic enzymes can effectively control the accumulation of process inhibitors which therefore allowed improvement desirable critical process attributes.

1. Introduction

Cell-based expression systems have been extensively used in the biomanufacturing industry to produce various biologics, of which production of therapeutic proteins remain among the most dominant segments in the healthcare industry [1]. Maximizing production capability of recombinant protein production to meet global healthcare market demand is therefore of paramount importance. Chinese Hamster Ovary (CHO) was the predominant mammalian expression platform of the recent approved biotherapeutics [2]. The ability to genetically engineered with recombinant DNA [3], the ease to grow at large scale in industrial bio-culture setting [4], and the biocompatibility for human-like post-translational modification [5], are all key drivers for bringing CHO to the forefront of the biotechnology industry in terms of host cells for therapeutics production.

Despite the advantages, industrial CHO bioprocesses often face challenges in lifting titer productivity to a higher ceiling, most commonly due to the inability to predict CHO cellular metabolism during later stage of the culture [6]. CHO cells are known to consume large amount of nutrients during growth and production. However, intracellular metabolism is poorly regulated which prevents cells to fully utilize nutrients to support growth and proteins production. Instead, significant fraction of fed glucose and amino acids are diverted into generation of metabolic by-products throughout different amino acids metabolism pathway. Traditionally, lactate and ammonia have been reported through literature to be the most dominant forms of by-products generated from glycolysis and other cellular metabolic cycles throughout the culture duration [7–9]. Studies have shown that accumulation of these by-products can inhibit cell growth and suppress peak viable cell density which can negatively impact glycosylation

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profile [10–12]. Owing to this, liquid chromatography-mass spectroscopy (LC-MS) has emerged as one of the most powerful set of tools for various metabolomic studies due to its capability of analyzing countless of metabolites and medium additives from a single sample [13-16]. These advances in metabolomic techniques have enabled identification of additional cell-generated growth inhibitory metabolites, other than lactate and ammonia [17-19].

In our most recent work, it has been shown that various metabolomic studies can be applied on CHO bioprocess to identify toxic metabolic inhibitors generated from CHO metabolism that can negatively impact growth, titer productivity and glycosylation profile [20-23]. Though the study successfully addressed cellular phenotypes due to accumulation of metabolites in CHO culture, however, the insight of their cellular metabolism, along with the appropriate control strategy to mitigate generation of such metabolic waste, remains unexplored. Other studies suggested that multi omics study and genetic engineering of recombinant genes can be employed to develop the appropriate control strategy for mitigation of metabolic wastes [24–28]. Although pathway identification and their related metabolic functionality have been previously studied in other mammalian systems, the extent to which genes are expressed, along with the impact of the generated intermediates on cellular activities including growth and protein production in CHO systems are not currently well studied.

Owing to this, this present work therefore sought to develop a control strategy using expression vectors to modulate the expression of recombinant genes related to metabolic pathways capable of metabolizing toxic inhibitors. By coupling genetic engineering with pathway analysis of CHO cells carried out through plasmid transfection, the study successfully showed that the accumulation levels of metabolic wastes, as quantified through off-line LC-MS measurement, were decreased in CHO extracellular environment. The study identified branched-chain amino acids (BCAA) metabolism genes *Adh5*, *Bcat1*, and *Hadhb* can effectively control the generation of inhibitors, which directly translated to improvement in higher cell growth, peak cell densities, and IgG1 titer production.

2. Materials and methods

2.1. Method development through pathway analysis

Screening of target genes was first conducted through batch mode cultivation of CHO cells under regular operating conditions (no perturbation). The concentration of key nutrients including essential amino acids and glucose accumulated/depleted in the extracellular environment throughout the culture duration was continuously measured via LC-MS, of which the respective metabolic uptake fluxes were calculated and used as input constraints in CHO-K1 metabolic model. Flux balance analysis (FBA) was then performed with the objective function to maximize biomass to establish a baseline assessment on growth and biomass productivity. Subsequently, each gene encoded in the gene library of the model was up-regulated which allowed re-assessment of the objective function at the end of each successful iteration. The outcome of the study observed various metabolic genes that showed improvement in productivity towards biomass, which suggested that metabolic fluxes have been efficiently re-allocated towards energy production for growth while decreasing the generation of wastes and metabolic by-products. Ultimately, by coupling computer-aid simulation work with the identification result obtained in reported publication where by-products metabolites were biologically confirmed to suppress growth in CHO culture, genes that were characterized to both contributing to the bioprocess by promoting growth in terms of biomass when compared to the established baseline, and also involving in biochemical processes that consumes the identified metabolites, were further considered for genetic engineering verification study.

2.2. Fed-batch process of CHO cells

A CHO-K1 cell line expressing IgG (VRC01) antibody from NIH (National Institute of Health) was used for this study with two proprietary media. For inoculation, basal medium (medium A) from MilliporeSigma (Burlington, MA, USA) and glutamine at 8 mM from Corning (Corning, NY, US) were supplemented to cell culture on Day 0. For fed-batch process, enriched nutrient feed medium (medium B) from Lonza (Portsmouth, NH, USA) was used as feed medium and fed to cells after Day 0 until cellular viability dropped below 80%. The initial feeding day was dependent on the development of feeding strategy and was addressed where appropriate throughout the discussion. Cells were cultivated in sterile 125 mL shake flask from Fisher Scientific (Waltham, MA, USA) with a working volume of 30 mL in a humidified, shaking incubator (Model AJ125) from ATR Biotech (Laurel, MD, USA) operated at 125 RPM, 36.5°C, and 5.2% carbon dioxide. For the fed-batch feeding study, a bolus of either 5%, 10% or 15% of the initial feeding volume was administered daily to cells. Cells were inoculated at low viable cell density (VCD) (approximately 0.5×10^6 cells/mL) in basal medium and harvested when cell viability decreased below 80%. Sub-culturing was done every two to three days when the cells reached VCD greater than 3×10^6 cells/mL. Cell viability was maintained above 90% throughout the inoculation phase.

2.3. Construction of expression vector

CHO-K1 cells were collected from Day 4 to Day 6 of fed-batch culture. Isolation and purification of CHO-K1 RNA was conducted using RNeasy Mini Kit from Qiagen USA (Germantown, MD, USA). Quantification of RNA concentration was performed on NanoDrop OneC instrument from Thermo Fischer Scientific (Waltham, MA, USA). Construction of CHO-K1 cDNA library was performed utilizing SS3 superscript kit from Invitrogen (Carlsbad, CA, USA). PCR amplification was conducted on the previously extracted cDNA library. The GOI included *Adh5*, *Bcat1* and *Hadhb*. Primers were designed based on the gene sequences obtained from NCBI database (see **Supplements,** Table 1) and cutting sites of plasmid restriction enzymes. PCR was conducted to amplify GOI using Q5® High-Fidelity DNA Polymerases from New England Biolabs (Ipswich, MA, USA). PCR products were purified with DNA gel electrophoresis at 100 V for eighty minutes and QIAquick Gel Extraction Kit from Qiagen USA (Germantown, MD, USA). Complex GOI/plasmid was constructed using GOI and pcDNA3.1/Zeo (+) plasmid from Invitrogen (Carlsbad, CA, USA). Complex GOI/plasmid was further digested with restriction enzymes from New England Biolabs. After dephosphorylation of 5′ end of plasmid using Quick CIP also from New England Biolabs, complex GOI/plasmid was ligated with using T4 DNA Ligase from New England Biolabs.

2.4. Bacteria transformation

Bacteria transformation was conducted using 50 μL of NEB® 5-alpha competent *E. coli* cells from New England Biolabs with 5 μL of previously ligated plasmids. Cells were mixed carefully by gently tapping on mixing tube five times and was incubated on ice for 30 minutes. Cells were heat shocked using a dry bath from Fisher Scientific (Waltham, MA, USA) to heat shock the cells at 42°C for thirty seconds. Cells were then incubated on ice for five minutes. A total volume of 950 μL of SOC Outgrowth Medium from New England Biolabs was added to the cells and the total mixture were transferred to 50 mL centrifuge tubes from VWR (Radnor, PA, USA). Tubes were slightly capped to allow cell respiration and incubated for ninety minutes in a humidified, shaking incubator from Queue Systems Inc (Columbia, SC, USA) operated at 250 RPM and 37°C. A total volume of 100 μL cells were uniformly and gently spread using a disposable inoculating loop from VWR onto a LB agar ampicillin plate from Sigma Aldrich (St. Louis, MO, USA) previously warmed to 37°C. Cells were incubated overnight at 37°C.

2.5. Plasmid propagation

Bacteria colonies (approximately three to four) were selected after the incubation period and were mixed with 5 mL of LB broth medium from Sigma Aldrich and 5 μL of ampicillin in 50 mL centrifuge tubes from VWR. Tubes were slightly capped to allow cell respiration and incubated for 12 hours in a humidified, shaking incubator from Queue Systems Inc operated at 250 RPM, 32°C. Plasmid extraction was performed using ZymoPURE II Plasmid Midiprep Kit from Zymo Research (Irvine, CA, USA) after twelve hours of incubation. Cell pellets were isolated by centrifuge from Eppendorf (Framingham, MA, USA). Plasmid concentration (in the range of ng/μL) was measured using NanoDrop from Thermo Fischer Scientific. For validation process, restriction enzymes were used to cut and validate the GOI followed by agarose gel electrophoresis.

2.6. Flow cytometry

CHO cells were obtained by screening at different time-points after the transfection. After 48 hours post transfection, the transfection efficiency and eGFP mean fluorescence intensity (MFI) of each condition was analyzed using a FACSCalibur cytometer from Becton Dickinson (Franklin Lakes, NJ, USA). Non-transfected cells were used as the negative control. A total of 100,000 fluorescent events were acquired using a 530/15 bandpass filter for the eGFP signal, which was obtained with fluorescence emission centered at 530 nm. After 7-day transfection, the MFI for each vector was measured again, and lower, medium, higher producers (% M1, M2, M3) and coefficient of variation (CV) of each sample were determined at the same time using a FACSCalibur cytometer from BD Biosciences (Bedford, MA, USA). All experiments were repeated three times.

2.7. Cells, media, and supplements

Cloning studies of metabolic genes were conducted on a CHO-K1 cell line expressing IgG (VRC01) antibody obtained from NIH (National Institute of Health) using a proprietary basal medium (medium A) obtained from Sigma Aldrich (St. Louis, MO, USA) and glutamine at 8 mM obtained from Corning (Corning, NY, USA) supplemented to cell culture on Day 0. Cells were cultivated in sterile 125 mL shake flask obtained from Fisher Scientific with a working volume of 30 mL in a humidified and shaking incubator (Model AJ125) obtained from ATR Biotech (Laurel, MD, USA) operated at 125 RPM, 36.5°C, and 5.2% carbon dioxide. Cells were inoculated at low viable cell densities (VCD) (approximately 0.5×10^6 cells/mL) in basal medium and harvested when cell viability decreased below 80%. Sub-culturing was done every two to three days when cells reached VCD greater than 3×10^6 cells/mL. Cell viability was maintained above 90% throughout the inoculation phase. Stock solution of each candidate metabolite was prepared by dissolving pure metabolite standards obtained from Sigma Aldrich into ultrapure water from a MilliQ water purifier obtained from MilliporeSigma (Burlington, MA, USA), and were supplemented in the basal media on Day 0 for both batch and fed-batch processes. For process validation, batch process of CHO-S cells was conducted in medium A for seven days.

2.8. Assessment of growth and productivity

VCD and viability were measured daily with a cell counter device (CeDex HiRes) obtained from Roche (Branchburg, NJ, USA). Glucose, lactate, and ammonia concentration data from each culture condition were measured using an analyzer (Bioprofile FLEX) obtained from Nova Biomedical (Waltham, MA, USA). Titer analysis was performed using a high-pressure liquid chromatography system (Agilent 1100 series) obtained from Agilent Technologies (Santa Clara, CA, USA) with a protein A column (Poros A, 2 μm, 2.1×30 mm) obtained from Thermo Scientific (Waltham, MA, USA). Cell culture supernatant samples were collected at different days for metabolomics analysis and titer was analyzed on the harvest day. The integral viable cell density (IVCD) at day *n* was defined as the summation of VCD for each day during CHO cell culture up until day *n*. To compare and evaluate the performance of each culture condition, the productivity at day *n* was calculated – defined as the overall measured titer (mg/L) normalized against IVCD at day *n*.

2.9. LC-MS samples preparation

LC-MS grade chemical solvents and reagents from MilliporeSigma were used as buffers for all LC-MS analysis. A standard solution containing 20 mM trans-aconitic acid, 320 μM leucinic acid, 64 μM methylsuccinic acid, 16 μM indole-3-carboxylic acid, 64 μM cytidine monophosphate, 64 μM guanosine monophosphate, and 3.2 μM N-acetylputrescinium in water was prepared. Serial dilutions with water produced working standard solutions. An internal standard (IS) solution containing 500 μM $^{13}C_6$-trans-aconitic acid, 200 μM $^{2}H_3$-leucinic acid, 20 μM $^{2}H_6$-methylsuccinic acid, 50 μM $^{2}H_5$-indole-3-carboxylic acid, 50 μM $^{15}N_3$-cytidine monophosphate, 50 μM $^{15}N_5$-guanosine monophosphate, and 5 μM $^{2}H_3$-N-acetylputrescinium was prepared in water. Each sample was prepared by mixing 10 μL of sample with a matrix containing 10 μL culture medium, 10 μL IS and 30 μL acetonitrile. Samples were vortexed for 30 seconds and centrifuged for 20 minutes at 16,000 g. The supernatant was then collected for LC-MS analysis.

2.10. LC-MS analysis methods

Analysis of cell culture sample was performed using 2 μL sample injecting to an ACQUITY UPLC instrument from Waters (Milford, MA, USA) coupled with a quadrupole time-of-flight Xevo G2-XS mass spectrometer also from Waters. For inhibitory metabolites analysis, LC separation was performed on a PEEK coated column (SeQuant ZIC-cHILIC, 3 μm, 100×2.1 mm) with a guard kit (SeQuant ZIC-cHILIC, 5 μm, 20×2.1 mm), both obtained from Millipore Sigma. The elution program was set at: 10% A (0–0.5 min), 30% A (2.5 min to 5.5 min), 45% A (5.5 min to 7.0 min), 65% A (7.0 min to 10.0 min), 10% A (10.1 min to 14.0 min). For MS analysis, mass range was set to 50–1200 Da with scan time of 0.5 second in centroid mode. Mass detection was performed in both positive and negative polarity under sensitivity mode. Leucine-enkephalin (leu-enk) was applied as lock mass reference for accurate mass calibration to counteract the potential effect of calibration drift during the long analytical sequences run time. For leu-enk lockspray setup, the following parameters were applied: positive mass 556.2771 Da; negative mass 554.2615 Da; scan time 0.5 seconds; scan interval 30 seconds; mass window ± 0.5 Da. For MS settings, the following parameters were used for electrospray ionization (ESI): capillary voltage 3.0 kV; sampling cone 40 V; source offset 80 V; source temperature 100°C; desolvation temperature 20°C; cone gas 40 L/hr; desolvation gas 600 L/hr.

2.11. Glycan analysis

Human serum IgG reference standard, 2-aminobenzamide (2AB), 2-picoline borane complex (2PB), ammonium formate and acetonitrile (HPLC-grade) were obtained from Sigma Aldrich (St. Louis, MO). Magnetic beads coated with protein A and protein G were purchased from Thermo Fisher Scientific (Waltham, MA). A peptide:N-glycosidase F (PNGase F) enzyme kit containing 10X denaturing buffer, NP-40 non-ion surfactant detergent and 10X reaction buffer was purchased from New England Biolabs (Ipswich, MA) and stored as instructed by the manufacturer. Diol solid phase extraction cartridges HyperSep™ Diol SPE were purchased from Thermo Fisher Scientific (Waltham, MA). Glycoclean™ S cartridges were from Prozyme (Hayward, CA). An inline filter (0.2 μm) was purchased from Restek Corporation (Bellefonte, PA) and a BEH amide column (1.7 μm, 2.1 mm×50 mm) was purchased from Waters Corporation (Milford, MA). For summary of a detail protocol,

interested reader can refer to a previously established protocol [29].

2.12. Growth and exchange rate calculation

Growth rates of CHO cells were assumed to follow exponential growth behavior:

$$N_x = N_{x,0} \bullet e^{\mu t} \tag{1}$$

Here $N_{x,0}$ ($\times 10^6$ cells/mL) is the number of cells at initial time, N_x ($\times 10^6$ cells/mL) is the number of cells after culture time t (hr). Rearranging (1), cell growth rate μ (hr^{-1}) therefore can be expressed as a log-based growth model:

$$\mu = \frac{\ln(N_x/N_{x,0})}{t} \tag{2}$$

During cellular expansion phase, it can be expected that cells follow closely to a linear growth model. Assuming CHO cells growing exponentially, the metabolite exchange rates r (mmol/hr) can be estimated by evaluating the change in measured concentration of metabolite i $[C_i]$ (mmol) over time:

$$r = \frac{d[C_i]}{dt} \tag{3}$$

Assuming CHO cells growing exponentially, r can be estimated by evaluating the change in C_i overtime:

$$r = \frac{dC_i}{dt} \approx \frac{\Delta C_i}{\Delta t} \tag{4}$$

The *IVCD* profile of cell at time t_n can be calculated as follows:

$$IVCD_{t_n} = IVCD_{t_{n-1}} + \frac{(VCD_{t_n} + VCD_{t_{n-1}})}{2}\Delta t \tag{5}$$

3. Results and discussion

3.1. Overall workflow of plasmid construction with restriction enzyme and genes cloning

Biological verification on the control strategy towards controlling the accumulation level of metabolites was done via transient transfection. An overall detailed genetic engineering pipeline for subclone development and gene functionality analysis employed in this study was shown in Fig. 1. In this metabolic genetic engineering approach, CHO-K1 cells are initially harvested from Day 4 to Day 6 from a fed-batch culture. CHO-K1 cells were initially harvested from Day 4 to Day 6 from a fed-batch culture. Cellular mRNA is isolated and purified to generate an RNA template of which CHO-K1 cDNA is reversely synthesized. DNA amplification is conducted by PCR to clone target GOI from cDNA library. PCR products are then collected and validated via agarose gel electrophoresis, from which fragments containing GOI are extracted and purified. Circular plasmid backbone with antibiotic resistance genes allowing individual selection of expression plasmids is digested with restriction enzyme to obtain linearized plasmid construct with sticky overhangs. For construction of recombinant plasmid DNA, DNA fragments of both GOI and linearized expression vector were ligated with DNA ligase. Host *E. coli* was then transformed with recombinant plasmid vector construct. Host transformants are selected via the integrated ampicillin resistance marker, after which transformants are recovered and further propagated to obtain plasmid in the concentration of approximately 40 μg/μL per GOI. The final engineered product plasmids with the desired structure are then harvested and further expressed in CHO systems for different study purposes. Overall, this hereby presented genetic engineering strategy enables rapid testing of genes functionality and metabolic impact due to their intrinsic capability of regulating the

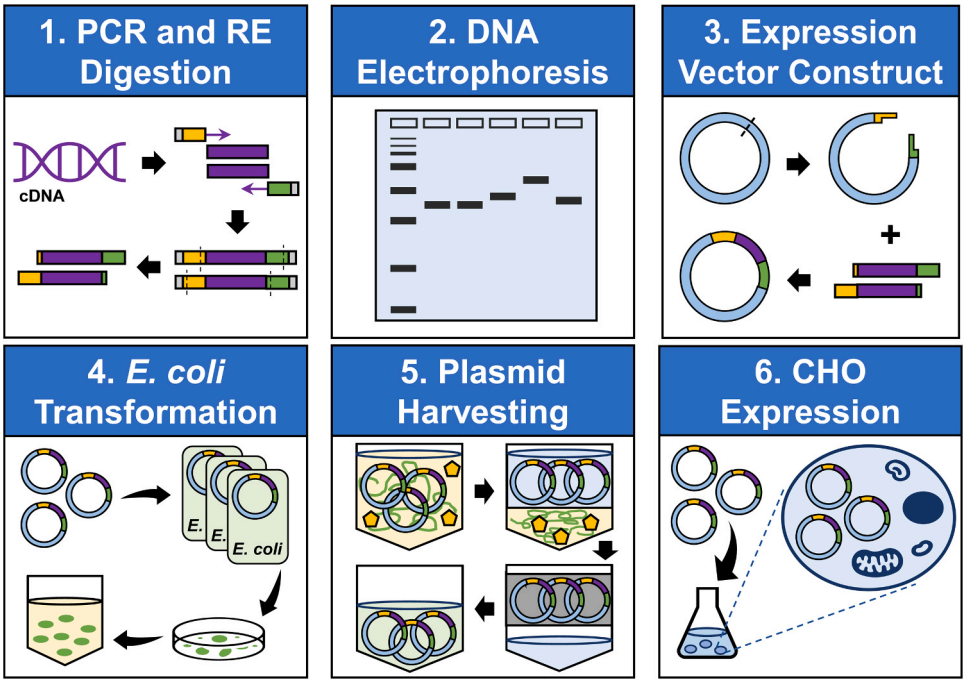

Fig. 1. Genetic engineering pipeline as employed in this study for subclone development and gene functionality analysis. In this study, cellular mRNA was isolated and purified to generate an RNA template of which CHO-K1 cDNA was reversely synthesized. DNA amplification was conducted by PCR to clone target GOI using a pair of forward and reverse primers strategically designed to ensure DNA amplicons containing in-frame expression of GOI and bearing appropriate restriction enzyme sites at both 5′ and 3′ ends. PCR products were collected and validated via agarose gel electrophoresis, from which DNA fragments containing GOI were extracted and purified. Circular plasmid backbone with different antibiotic resistance genes for individual selection of expression plasmid is digested with restriction enzyme to obtain linearized plasmid construct. For construction of recombinant plasmid DNA, DNA fragments of both GOI and linearized expression vector were ligated with DNA ligase. Host *E. coli* was then transformed with recombinant plasmid vector construct. Host transformants are selected via the integrated ampicillin marker, after which transformants are recovered and further propagated to obtain plasmid in the concentration of approximately 40 μg/μL per GOI.

accumulation level of metabolic waste inhibitors.

3.2. Pathway analysis of CHO metabolism

Previous study conducted in our group applied global metabolomic analysis on different modes of CHO bioprocess to identify eight different growth by-products accumulated throughout the entire culture duration of cells. Although growth inhibitory effects due to presence of these metabolites in CHO extracellular environment has been verified, the corresponding pathway and the extent through which their nutrient precursors are metabolized currently remains unknown, which poses difficulties in development of control strategies to decrease the accumulation of metabolic inhibitors. Here, a strategic methodology was developed via pathway analysis coupled with GEM to control the extent through which growth inhibitory metabolites are accumulated in CHO bioprocess. Metabolic pathway analysis was conducted on each metabolite via GEM, after which metabolites were mapped to a holistic regulatory metabolic network. First, all metabolic enzymes capable of either direct/indirect catabolizing of toxic metabolites or re-allocating metabolic fluxes into alternative metabolic branches not generating by-products inhibitors are considered. The proposed platform sought to control the accumulation of inhibitors by up-regulating the expression of identified candidate genes responsible for modulation of such enzymes. The list of candidate genes was further biologically verified through genetic engineering work for assessment of the outcome impact on growth. The final list of genes through which up-regulation of enzymatic activities can promote production towards growth, the related metabolic pathways, and the corresponding input metabolites are shown in Fig. 2 A.

3.3. Plasmid construction with restriction enzyme and genes cloning

To ensure proper expression of target GOI, forward and reverse primers were strategically designed to ensure DNA amplicons bearing appropriate 5′ and 3′ ends. Specifically, DNA sequence of each target GOI was reviewed from the literature, of which the introduced restriction sites as found on the primers remained unique and not presented within the sequence of the fragment to be subcloned (see Fig. 2 B). Additionally, primers were also designed to contain the expressed sequence as the open reading frame to ensure proper in-frame expression. Spacer sequences were introduced at the 5′ end of the primer sequences to ensure proper binding and digestion of the template fragments. Construction of plasmid vector was carried out with pcDNA3.1 backbone with multiple cloning site region in the forward position, hereby denoted as

A

GOI	Metabolic Pathways	Targeted Metabolites
Adh5	Leucine Metabolism	HICA, MSA
Bcat1	Leucine and isoleucine metabolism	HICA, MSA
Hadhb	Isoleucine metabolism	HICA, MSA

B

GOI	Direction	RE	Primer Sequence 5′ - 3′
Adh5	Forward	AflII	TAATATATACTTAAGGCCACCATGGCGAACCAGGTGAT
	Reverse	EcoRI	CGGCCGGAATTCTTACAGCTTTAGAACAGT
Bcat1	Forward	AflII	TAATACTTAAGGCCACCATGGACTGCAGTAAT
	Reverse	EcoRI	GCCCGAATTCTCACGATAACAAGATTG
Hadhb	Forward	AflII	TATACTTAAGGCCACCATGTCTACCATCTTGACTT
	Reverse	EcoRI	CCGGAATTCTCATTTTGGATAAGCTTCC

C

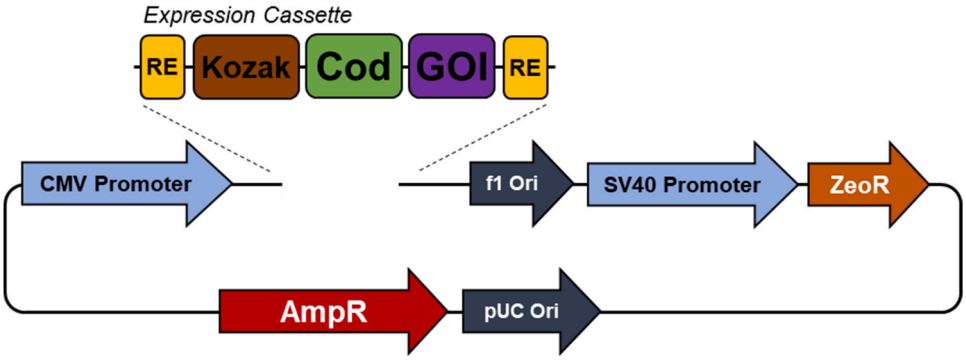

Fig. 2. Cloning of different metabolic gene of interest (GOI) into vector construct assembly. (A) Genes, corresponding metabolic pathways and regulated downstream metabolites (B) Target gene of interest (GOI) flanked by restriction enzymes (RE) along with the corresponding forward and reverse primers as cloned through PCR amplification. (C) Blueprint of vector construct assembly. For (B), to ensure the expression of the GOI, Kozak sequence was engineered into the expression cassette to promote translation activity along with the codons. Different metabolic GOI native to CHO genome were cloned into circular plasmid vector construct for study of their enzymatic activities to control toxic metabolites accumulation when up-regulated in CHO host. Restriction enzyme cutting sites found on the 5′ ends of both forward and reverse primers are unique and not present within the sequence of the fragment to be subcloned. Primers are also designed to contain the expressed sequence as the open reading frame to ensure proper in-frame expression. Spacer sequences are introduced at the 5′ end of the final primer sequence to ensure proper binding and digestion of the template fragments.

pcDNA3.1/Zeo(+). Inclusion of ampicillin resistance marker and pUC replicon allowed selection for high cell density propagation when transforming into *E. coli* host. To ensure the expression of the GOI, a Kozak sequence was engineered into the expression cassette to promote translation activity. The key to this strategy is the complementary overhangs generated by restriction enzyme cleavage (5′ *Afl*II and 3′ *Eco*RI) that allow for insertion of the Kozak-GOI complex as the open reading frame to the acceptor site located on the plasmid backbone (see Fig. 2 C). This plasmid toolkit as described here was used in subsequent study for analysis of gene expression level and metabolic functionality.

3.4. Engineering strategy targeting the accumulation of metabolites

Pathway analysis conducted on HICA and MSA revealed their pathway of origin stemming from the catabolism of BCAA. In the first step of the catabolism, transfer of an α-amino group from each of the precursor amino acid (leucine, isoleucine, and valine) yields three different respective branched-chain α-ketoacids [30–32]. Deamination of leucine is catalyzed by amino acid transferase to transfer the amino group in leucine to α-keto acid acceptor in α-ketoglutarate. Other BCAA (e.g., valine and isoleucine) also share the same property of being α-keto acid acceptor as leucine [33]. Further analysis revealed HICA to be the end-product of the leucine metabolism, specifically through the direct conversion of keto acid into hydroxyl acid. Therefore, regulation over the accumulation of α-ketoisocaproic acid (KIC) serves as the main strategy in modulating metabolic fluxes into formation of HICA, as illustrated in Fig. 3. Specifically, by re-distributing the fluxes converting α-ketoglutarate to glutamate to other BCAA would effectively decrease the presence of α-ketoglutarate at a given time that can participate in the conversion of leucine to KIC. Therefore, up-regulation of cytosolic BCAA transaminase (*Bcat1*) was expected to be an effective approach to control HICA generation. An argument could be made where reducing KIC could compensate the fluxes available going into the generation of acetyl-CoA, which is a crucial substrate for energy generation throughout the Krebs cycle. While that remains true regarding the amount of acetyl-CoA generated by catabolism of leucine, it is important to note that acetyl-CoA can also be generated from the catabolism of isoleucine, or from the conversion of succinyl-CoA, and thus down-regulating the degradation pathway of leucine would generate less amount of HICA metabolites as by-products.

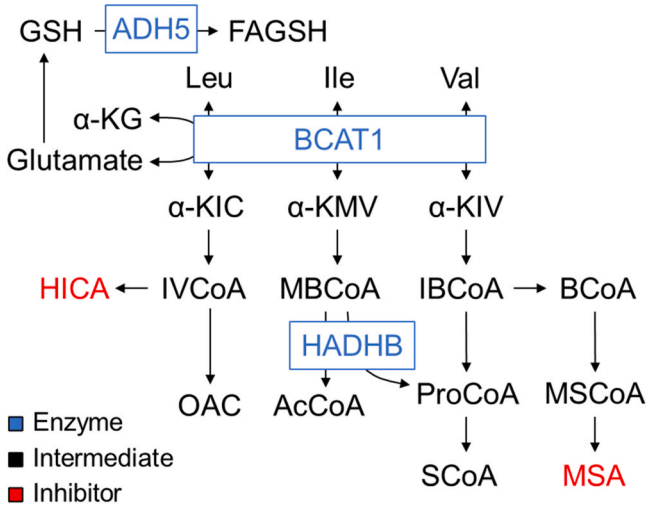

Fig. 3. Genetic engineering strategy targeting accumulation of α-hydroxyisocaproic acid (HICA) and methylsuccinic acid (MSA) accumulation in CHO cells. In this study, metabolic by-products HICA and MSA previously identified to be growth inhibitors accumulated from a batch and fed-batch CHO bioprocess were targeted for genetic engineering study. Genome scale modeling (GEM) revealed additional metabolic pathways that can be controlled through genetic flux modulation. Accumulation of inhibitors can be controlled by up-regulating the expression of characterized genes responsible for modulation of metabolic enzymes that catalyze reaction.

Additional pathways were also studied via genome-scale modeling. In CHO cells, alcohol dehydrogenase (ADH5) catalyzes the extracellular transport reaction involving formaldehyde and glutathionate. Investigation into glutathione through GEM reveals key reactions mapping glutathionate to KIC via the leucine metabolism (see Fig. 3). Specifically, glutathionate participates in the reaction generating cysteine from cystine which is a by-product from the transporting reaction of allocating cytoplasm leucine to extracellular leucine. Additionally, through the glutathionate metabolism, glutamate is generated which serves as a participating metabolite in the generating reaction of KIC. Up-regulation the activity of ADH5 further metabolizes glutathionate into formaglutathionate, reducing the amount of glutathionate available for participation in other reactions generating complimentary substrates (decreasing the amount of leucine generated which can metabolized into KIC or decreasing the amount of glutamate which can catalyze the reaction generating KIC).

3.5. DNA electrophoresis and plasmid transfection efficiency assessment as validation tools

DNA electrophoresis was employed in this study to validate recombinant DNA plasmid construct, as shown in Fig. 4 A. Specifically, plasmid constructs were treated with the same pair of restriction enzyme previously used to cut CHO synthesized cDNA to form sticky overhangs. The key to this strategy lies in the ability to validate both the recombinant plasmid post ligation as well as the target engineered GOI on a single gel slab. Besides the plasmid backbone which remained unchanged across several types of plasmid construct, both the recombinant plasmid and individual GOI were shown to migrate to distinct locations as found in the DNA electrophoresis study, further confirming the intact plasmid construct as engineered in this study.

Next, this work sought to validate the expression of recombinant plasmid in mammalian host. Here, GFP gene was employed as reporter gene for verification of expression during translation to assess the transfection efficiency. Plasmid engineered with GFP gene was inserted into CHO-S cell line and the fluorescence intensity was validated through both fluorescence microscopy and flow cytometry. A wild-type condition was added as the negative control to the transfected conditions. Control cells and transfected cells were both derived from the same culture to eliminate variation in growth cycle and other culture conditions. Comparison of cellular physiology post-transfection on Day 2 and Day 3 between the transfected and control condition as measured through flow cytometry is shown in Fig. 4 B. In the study, CHO-S cell lines showed 35.2% transfection efficiency on Day 2 as cultivated in the supplied media and process. On Day 3, flow cytometry results indicated measured efficiency of 43.1%, further demonstrating that cells have successfully expressed GFP during CHO-S production period, suggesting an exhibited peak protein production rate between Day 2 to Day 3 of cells cultivated in batch process. The result of the study was also confirmed through fluorescence microscopic imaging, as cells also exhibited high degree of fluorescence marker when visualized under fluorescence light, as illustrated in Fig. 4 C.

3.6. Quantification of expression level of transfected genes through qPCR

The expression level of transfected metabolic genes as expressed in CHO-S cells were evaluated using relative quantification (RQ) compared to the calibrator. To study metabolic genes functionality towards growth and protein synthesis, a new batch process of CHO cells was conducted. Here, the study was divided into three unique subclone groups, where each subclone was overexpressed with an individual GOI via transient transfection at plasmid concentration of 40 μg/μL to cells. A negative control was included in the study where no genetic engineering work

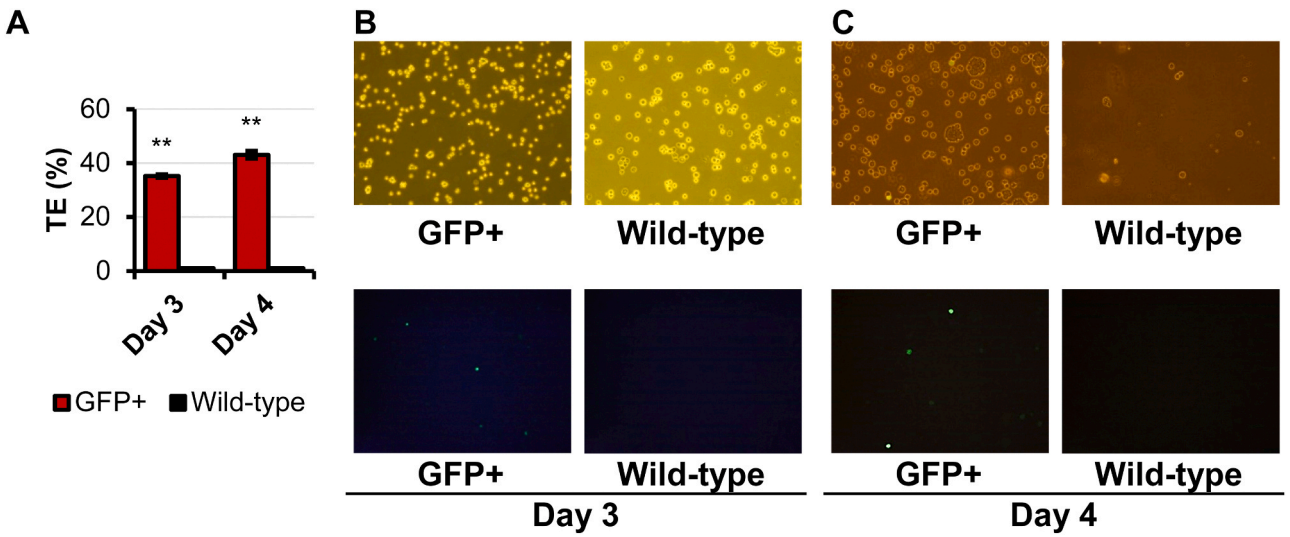

Fig. 4. Validation of recombined plasmid construct engineered with target gene-of-interest (GOI) and protein expression level. (A) Transfection efficiency (TE) of CHO-S cells as measured through flow cytometric assay on Day 3 and Day 4 post transfection. Imaging of CHO-S cells under normal light and fluorescence light during peak protein production period of cells cultivated in batch process on (B) Day 3 and (C) Day 4. In this study, GFP reporter gene was used as indicator for assessment of translation activity. Cells transfected with GFP-inserted plasmid (GFP+) were compared against wild-type cells (no GFP) to evaluate expression of GFP reporter protein. CHO batch process was conducted at 0.5×10^6 cells/mL seeding density in 30 mL culture volume.

involving plasmid transfection occurred. Cells from each group were derived from the same parental culture to eliminate variation in growth cycle and other culture conditions. Each subclone was cultured for 6 days. Cells were grown to confluency (Day 3) and were transfected with plasmids containing different GOI depending on each condition. The expression levels of engineered metabolic genes in CHO cells were quantified via qPCR and normalized against the control, as shown in Fig. 5 B-D. Overall, the quantified expression levels of all transfected genes from all mutant conditions were found to be higher when compared against the control, further confirming proper translation and expression of target GOI when transfected to host cells. From the results of the study, it was clear that the gene expression level from the wild-type control condition was the lowest in all cases, as compared to 40 μg/μL of plasmid concentration as found from the transfected conditions. The range of the obtained RQ values showed considerable variability among each different transfected condition when compared against each other. Thus, the study suggested that all engineered plasmids, although individually might exhibit a different level of gene expression when inserted into cells depending on the GOI and its impact on cellular metabolism, all show higher level of gene expression versus the wild-type condition from cells obtained on Day 3 where the gene expression levels of cells cultivated in batch process are usually at peak, further indicating that plasmids were successfully transfected into cells and all GOI engineered in plasmid vectors were properly expressed during the cellular translation process.

3.7. Assessment of culture performance

Critical process performance (CPP) in terms of cellular phenotypic behaviors such as VCD profile, IVCD profile and growth rate obtained from conducting batch process were systematically evaluated to assess the impact of up-regulating metabolic activities due to genes over-expression across all tested conditions. In general, all studied conditions showed promoted cellular phenotypes when compared against the control (see Fig. 5 E to G). For all engineered conditions, the peak VCD profile of cells on Day 4 realized a 18–20% increase ($7.3–7.5\times10^6$ cells/mL) when compared against the control (6.1×10^6 cells/mL). Similarly, the cumulative VCD profile also realized 16–19% increase ($35.6–36.7\times10^6$ cells/mL) in terms of total viable cells on Day 6 versus the control (30.3×10^6 cells/mL). Equivalently, higher VCD profile also translated to better growth rate profile of cells following the transfection date as calculated by a log-based growth model (see Fig. 5 G). Overall, improvement in cellular performance throughout the entire culture duration (Day 0 to Day 6) with respect to the target product CPP was realized across all studied conditions. The conducted study suggested that the metabolism of cells have been better regulated by confirming a decrease in the generation inhibitory by-products; and thus, by effectively up-regulating metabolic activities capable of consuming harmful inhibitors generating into the spent medium, the impact of these identified rate-limiting factors was effectively mitigated, allowing cells to achieve a higher growth rate and better overall peak and cumulative cell densities. The results altogether suggested that CHO cell growth in bioprocesses can be improved, either by i) increasing the generation of cellular energy utilized towards growth by metabolizing growth inhibitory metabolites as reactants in some understudied biochemical pathways (and therefore reducing their accumulating concentration) or ii) re-distributing metabolic fluxes into alternative metabolism pathways that do not generate metabolic by-products that can suppress cellular proliferation.

3.8. Genes functionality study through metabolic pathway analysis

A major challenge of mammalian bioprocess is the generation of unwanted toxic by-products during growth and protein production phase of cell culture. As briefly mentioned earlier, metabolite HICA is generated as the downstream metabolite from the metabolism of leucine, of which their cellular biochemical activities can be regulated either through modulation of upstream ADH5 and BCAT1 or downstream HADHB enzymatic activity. In this work, the concentration of various metabolic inhibitors accumulated in the spent medium at harvest day as generated from all studied genetically engineered subclones were analyzed. In summary, control of HICA as generated from CHO metabolism was realized at 86.13% (*Adh5*), 72.31% (*Bcat1*) and 27.27% (*Hadhb*) reduction in terms of measured concentration across all engineered subclones (see Fig. 5 J). With regards to the metabolism of all BCAA, valine metabolism pathway which generates toxic metabolite MSA can actively interact with both leucine and isoleucine neighbor pathways via different oxidized fatty acids (see Fig. 3). By up-regulating enzymatic activity of ADH5, BCAT1 and HADHB in cells, the study showed that improvement in MSA accumulation of CHO cells, as

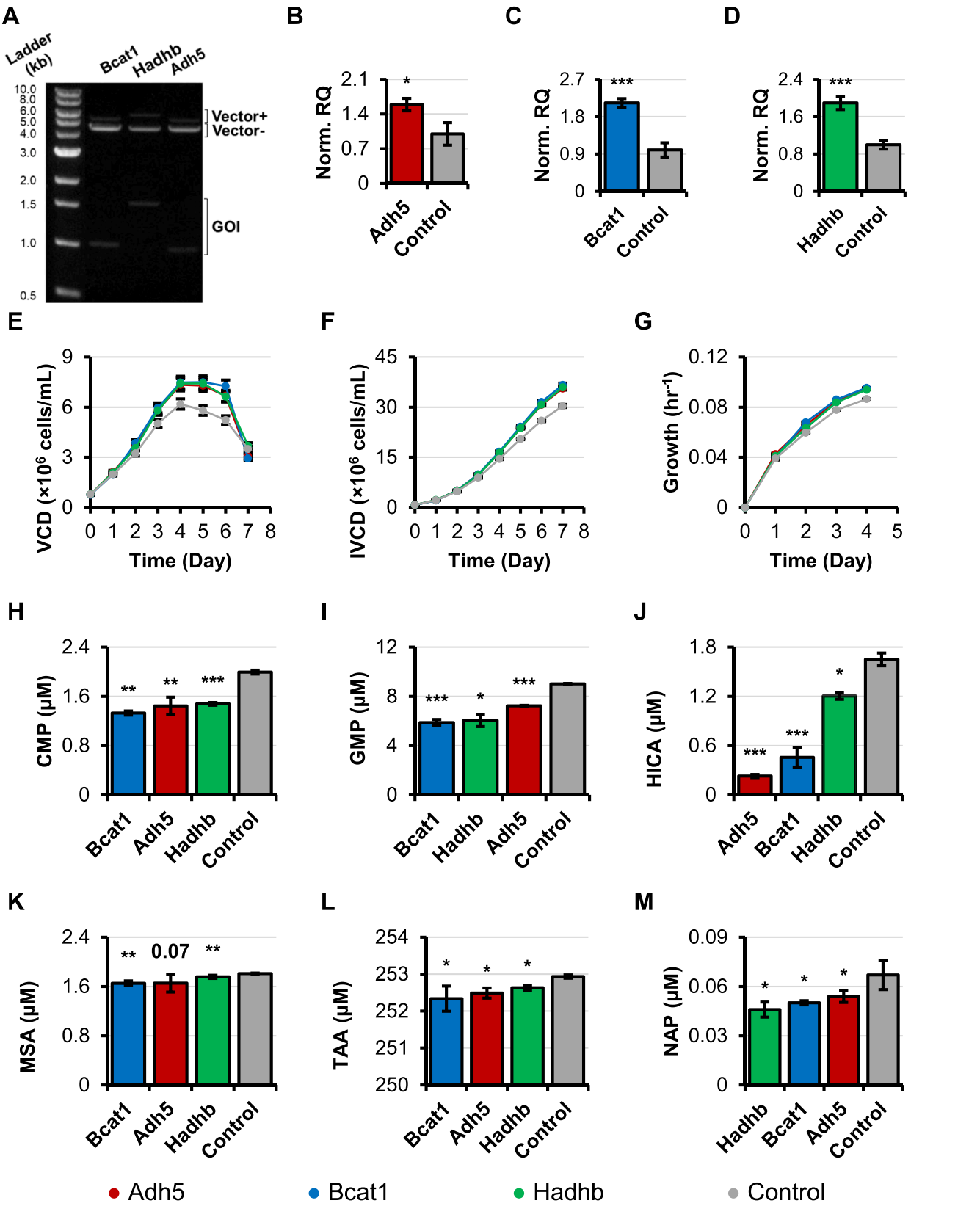

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Fig. 5. Gene expression level, cellular phenotype characteristics, and inhibitory metabolites accumulatio of genetically engineered CHO-S subclones. (A) DNA electrophoresis result of recombinant plasmid assembly. Gene expression level of (B) *Adh5*, (C) *Bcat1* and (D) *Hadhb* versus the control on Day 3 measured as relative quantification (RQ) through qPCR. (E) VCD profile. (F) IVCD profile. (G) Growth rate. Inhibitory metabolite accumulation profile: (H) CMP, (I) GMP, (J) HICA, (K) MSA, (L) NAP, and (M) TAA. For (A), each type of plasmid construct was treated with the same pair of restriction enzyme previously used to cut cDNA to evaluate GOI and linearized vector. Treating of fully constructed vectors with RE can yield three possible outcomes: single lone GOI, empty vector (Vector-), or full vector with intact GOI (Vector+). For (B) to (G), the study was divided into three unique subclone groups. Each subclone was overexpressed with an individual gene of interest via transient transfection at plasmid concentration of 40 μg/μL to cells. For (H) to (M), quantification of inhibitory metabolites accumulation was performed through LC-MS. Batch process of cells was conducted at 0.7×10^6 cells/mL seeding density in 30 mL culture volume. A negative control was included in the study (no plasmid transfection occurred). Cells were cultured until cell viability dropped below 80%. Cells for both the control and transfected conditions were derived from the same culture to eliminate variation in growth cycle and other culture conditions. Statistics was performed using two-tailed t-test. Shown here: bars (mean ± s.d., n = 3); * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$).

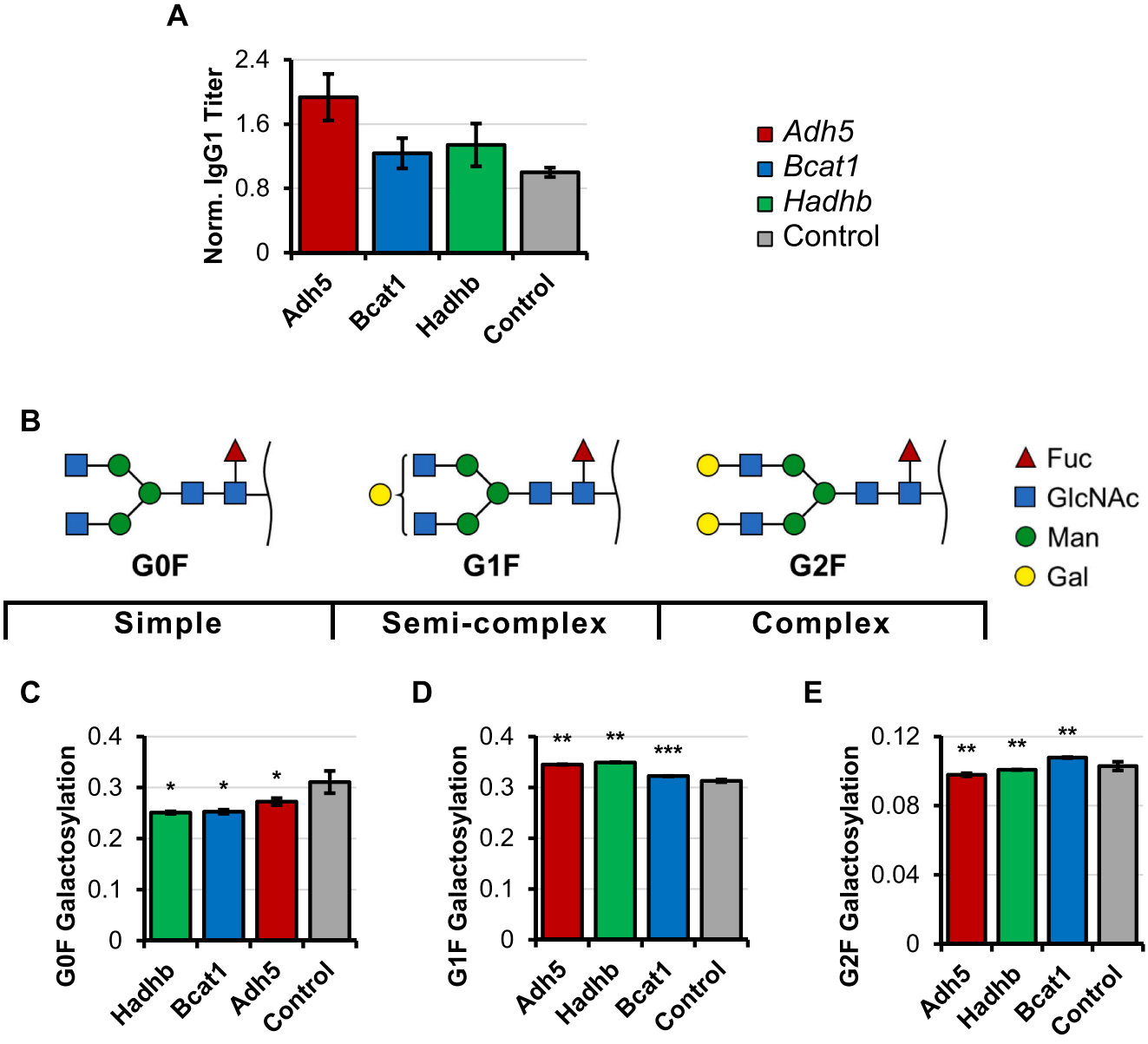

Fig. 6. IgG1 titer and glycoslyation profile of genetically engineered CHO cells. (A) Normalized titer profile obtained from each condition against the control. (B) Representative scheme of different glycan structures including a-galactosylated (G0F), mono-galactosylated (G1F), and bi-galactosylated (G2F) biantennary being investigated in the study. Comparison of different glycoform profiles including (C) G0F, (D) G1F, and (E) G2F glycans obtained from purified IgG1 secreted by transfected cells against the control. In this study, batch process of CHO cells was conducted at 0.7×10^6 cells/mL seeding density in 30 mL culture volume. The study was divided into three unique subclone groups. Each subclone was overexpressed with an individual gene of interest via transient transfection at plasmid concentration of 40 μg/μL to cells. A negative control was included in the study (no plasmid transfection occurred). Cells were cultured until cell viability dropped below 80%. Cells for both the control and transfected conditions were derived from the same culture to eliminate variation in growth cycle and other culture conditions. Bars, mean ± s.d.; n = 3, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Statistics by two-tailed t-test.

indicated by a reduction of MSA ranging from 3% to 10% across all genetically engineered conditions. This improvement is rather modest, particularly when compared to the significant decreases as observed in HICA (see Fig. 5 J). This could be attributed to MSA being a downstream metabolite, while ADH5, BCAT1, and HADHB are upstream enzymes. Thus, the impact of gene overexpression on MSA levels was relatively minor (as also illustrated – see), and therefore the effect of gene overexpression felt by MSA was relatively minor. Future studies can be conducted to confirm this finding.

The accumulation pattern of growth and productivity inhibitors other than HICA and MSA was also profiled (see Fig. 5 H to M). Interestingly, metabolites CMP, GMP, NAP, and TAA all showed decreased accumulation concentration as measured from all engineered subclones when compared against the control. The study also showed that in all cases, overexpressing key metabolic genes related BCAA metabolism can positively contribute towards decreasing the accumulation profile of metabolite CMP and GMP, further suggesting that pyrimidines are, in fact, interacted with many different intracellular pathways of metabolizing of amino acids. Altogether, the conducted study verifies that HICA and MSA are metabolic by-products generated through the metabolism of BCAA in CHO system comparable with other mammalian host cells. Additionally, the data also suggested that ADH5, BCAT1 and HADHB enzymes, although not directly participate in metabolic pathways native to the generation of certain process inhibitors, can potentially interact and catalyze various secondhand reactions that can directly influence over the fate of toxic inhibitors production via some understudied metabolic pathways. Finally, through genetic engineering work by overexpressing of upstream genes that involved in different metabolic pathways, the study also showed that up-regulation of the translation activity of metabolic enzymes, including ADH5, BCAT1, and HADHB can control the generation of growth inhibitory metabolites, either by increasing the fluxes of biochemical reactions that metabolize waste by-products (downstream enzyme to the generation of waste), or re-allocating the available metabolic fluxes into other alternative metabolic pathways.

3.9. IgG1 antibody and glycosylation profile

IgG1 antibody profile and glycosylation patterns of harvested titer obtained from transfected CHO cells obtained from the transfected cells were also shown in Fig. 6 A to E. One notable observation was that cells exhibiting up-regulated gene expression displayed a significantly enhanced titer profile compared to the control group. Specifically, there was a 1.1-fold increase in titer for *Adh5*, a 0.4-fold increase for *Bcat1*, and a 0.5-fold increase for *Hadhb*. The study also sought to investigate the glycosylation patterns as secreted by engineered CHO cells. Comparison of glycoform profiles obtained from purified IgG1 from both transfected cell groups were shown in Fig. 6 C to E. In this study, three different types of protein glycoforms were investigated, including simple glycan G0F, semi-complex glycan G1F, and complex glycan G2F (see Fig. 6 B). A predominant formation of G1F and G2F glycoforms suggests an improvement in bioprocess efficiency, indicating the cells enhanced capability to produce a higher fraction of semi-complex and complex glycoforms as opposed to the simple forms. Across all engineered strains, the study observed elevated levels of G1F and G2F glycans, coupled with a reduced presence of G0F when compared against the control. Intriguingly, our data revealed that up-regulating the *Adh5* and *Hadhb* gene significantly enhanced G1F glycosylation, while up-regulating the *Hadhb* gene had a similar effect on G2F, (see Fig. 6 D and E). The study suggests that modulating the activity of BCAA metabolism, either directly or indirectly, can beneficially influence the N-glycan biosynthesis pathway. Looking ahead, incorporating techniques like ^{13}C-aided metabolic flux analysis to examine glycoprotein formation via alteration in key enzyme expressions may provide deeper insights into the dynamic interplay between CHO cell metabolism and glycosylation profiles in mammalian cell culture processes. The results as obtained here, coupled with the previously shown decreased toxic metabolites profile as shown earlier, demonstrated that cell growth profile together with titer increase with better CQAs can be directly achieved through genetic engineering. We demonstrated the directionality of the research, also suggested that further optimization, such as using better-optimized vectors for higher expression, could potentially lead to an even more improved titer outcomes.

4. Conclusion

CHO cells are known to secrete growth inhibitory metabolites during growth and production phase, which hampers cellular performance and negatively impacts final productivity and product quality attributes. In this study, three different metabolic genes (*Adh5*, *Bcat1* and *Hadhb*) were cloned from CHO reversely synthesized cDNA to optimize cellular metabolism. Each metabolic gene was individually engineered to expression vector and transfected to cells at plasmid concentration of 40 μg/μL per gene. The study showed that by overexpressing *Adh5*, *Bcat1* and *Hadhb*, the concentration of inhibitory metabolites from the transfected subclones can be effectively controlled as measured through LC-MS. Improvement in cellular VCD, cumulative IVCD and growth profile of cells post transfection were also observed when compared against the control. In addition, an increase in titer production and more complex glycoform profiles were also realized from the engineered subclones. The study hereby successfully presented a control strategy that incorporated genetic engineering and inhibitory metabolites pathway analysis to generate a clean CHO process, suggesting genetic engineering of cells targeting key metabolic enzymes can improve performance of CHO cultures, with applications ranging from medium optimization, cells line engineering and process development.

CRediT authorship contribution statement

Bingyu Kuang: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Duc Hoang:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Seongkyu Yoon:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **George Liang:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Qiang Fu:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **SoYoung Park:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bej.2024.109282.

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