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RESEARCH ARTICLE

Cell Culture and Tissue Engineering

Trace metal optimization in CHO cell culture through statistical design of experiments

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Abstract

A majority of the biotherapeutics industry today relies on the manufacturing of monoclonal antibodies from Chinese hamster ovary (CHO) cells, yet challenges remain with maintaining consistent product quality from high-producing cell lines. Previous studies report the impact of individual trace metal supplemental on CHO cells, and thus, the combinatorial effects of these metals could be leveraged to improve bioprocesses further. A three-level factorial experimental design was performed in fed-batch shake flasks to evaluate the impact of time wise addition of individual or combined trace metals (zinc and copper) on CHO cell culture performance. Correlations among each factor (experimental parameters) and response variables (changes in cell culture performance) were examined based on their significance and goodness of fit to a partial least square's regression model. The model indicated that zinc concentration and time of addition counter-influence peak viable cell density and antibody production. Meanwhile, early copper supplementation influenced late-stage ROS activity in a dose-dependent manner likely by alleviating cellular oxidative stress. Regression coefficients indicated that combined metal addition had less significant impact on titer and specific productivity compared to zinc addition alone, although titer increased the most under combined metal addition. Glycan analysis showed that combined metal addition reduced galactosylation to a greater extent than single metals when supplemented during the early growth phase. A validation experiment was performed to confirm the validity of the regression model by testing an optimized setpoint of metal supplement time and concentration to improve protein productivity.

KEYWORDS
design of experiments (DOE), monoclonal antibody, productivity, statistical analysis, trace metal

1 | INTRODUCTION

Biotherapeutics have revolutionized modern medicine since their introduction in the 1980s. Majority of the biotherapeutics industry today relies on the manufacturing of monoclonal antibodies (mAbs) from Chinese hamster ovary (CHO) cells. In fact, due to their safety and efficacy as hosts for protein post translational modifications, CHO cells produce more than 60% of biologics in the market today.[1] With the market

for recombinant therapeutic proteins valued at over $170 billion in 2020 and projected to reach $450 billion by 2028,[2,3] these drugs are in high demand as the prevalence of chronic illnesses and infectious diseases across the globe continues to rise. Research and development efforts over the past few decades have significantly improved production yields yet challenges remain when it comes to maintaining consistent product quality from high-producing CHO cell lines. One popular approach to improving product yield is to leverage biological knowledge of the cells' microenvironment to control the rate of protein production as well as how the protein is modified during the bioprocess. The cell culture medium is a dynamic component to the cell culture process, which can be modulated in various ways to change process performance. Levels of relative trace metals are one category of media components that could be leveraged to alter protein production or modulate product quality attributes. Trace metals are substantial components in cell culture media that have been shown to impact CHO metabolism, growth, mAb production, as well as product quality attributes.[4–8] Variability even at ppb or micromolar level in raw materials during CHO fed-batch processes can influence these changes in cellular metabolism as well as cause significant product quality inconsistency. For example, zinc has shown to increase protein titer in recombinant CHO cell lines.[8,9] Some effects of metals on CHO cells seem to be cell line-dependent as well, such as the impact of iron on protein titer, glycosylation, cell growth, and nutrient profiles across CHO-K1 and CHOZN cell lines.[10] Furthermore, different counter-ion sources (i.e., ferric citrate versus ferric ammonium citrate to supplement iron) can lead to different cell culture outcomes[10] which needs to be considered. The effects of trace metals on cell culture performance can also depend on the availability of other nutrients in the cell culture medium, including other trace metals.[11] Previous studies have explored the impact of individual metals, such as iron, zinc, copper, or manganese on CHO cell metabolism, productivity, and product quality.[5,8,10,12,13] However, few studies to date explore the benefits of balancing the relative levels of trace metals within the media at certain times during cell growth to establish superior performance.

This study investigates the effect of zinc and copper supplementation on a CHO-K1 cell line by conducting a design of experiments (DOE) where zinc and copper supplements were spiked into CHO cell culture at various time points. Cell culture performance data was inputted into a statistical regression model to identify the most significant factors (and interactions of factors) contributing to the changes observed in cell growth, antibody titer, as well as other metabolic changes. The DOE identified a combined ratio of zinc and copper which when added at the time of inoculum improved antibody titer up to 77%. Changes to glycan profiles in response to the DOE conditions tested were also examined.

2 | MATERIALS AND METHODS

2.1 | Cell culture conditions and supplements

A VRC01 CHO-K1 cell line was used in all cell culture experiments. Prior to inoculating fed-batch cultures, CHO-K1 cells were seeded and expanded for three passages in a proprietary basal media supplemented with glutamine. Fed-batch cultures were inoculated at a seeding density of 2.5E+05 with 100 mL working volume in 250 mL shake flasks (Corning, NY) in an incubator maintained at 37°C, 125 rpm, and 5% CO_2. The metal supplements used were zinc sulphate heptahydrate (Sigma Aldrich, Milwaukee, WI) and copper sulphate pentahydrate (Sigma Aldrich, Milwaukee, WI) at 100 or 200 μM concentrations, added at either 0-, 60-, or 120-h post-inoculation. All conditions were run in fed-batch mode, where 10% working volume of proprietary feed media added when glucose level dropped below 2 g/L. To establish a base-line control condition for CHO-K1 cell, three additional fed-batch runs were conducted without metal supplement to determine the expected cell growth and nutrient profile. All other conditions were performed in duplicate or triplicate. Viable cell densities were measured daily using a Cedex HiRes Analyzer automated cell counter (Roche Life Science, Indianapolis, IN) based on a trypan blue dye exclusion assay. Daily metabolites (glucose, glutamine, lactate, and ammonia) were measured from cell culture supernatants using a Nova BioProfile Flex Analyzer (Nova Biomedical, Waltham, MA).

2.2 | Media characterization

Initial concentrations of zinc and copper in the fresh basal and feed media were measured using Inductively Coupled Mass Spectrometry (ICP-MS) (Agilent 7900). Sample preparation and analysis were based on a validated method developed by Mohammad et al. 2019.[14] Media samples were prepared by digestion in 2% v/v nitric acid, heated to 90°C, and spun down at 3500 rpm for 5 min prior to analysis. Initial zinc concentration in the basal media was ~16 μM and copper was ~0 μM (below the limit of detection of 1 μM), while the feed media contained ~60 μM zinc and ~ 2 μM copper.

2.3 | Experimental design

A 3-level factorial DOE was employed to assess implications of introducing individual or combined metal supplements at different culture stages of cell growth. The DOE was generated using MODDE Pro 13 (Sartorius Data Analytics), using a Box-Behnken design (BBD) where zinc and copper are supplemented in a time-wise fashion so that metabolic changes occurring during exponential cell growth and onset of the stationary phase can be deciphered. In this type of response surface methodology (RSM) experimental design, the primary interest is to fit a second-order regression (quadratic) model, where three levels (low, mid, and high) are tested for each factor. The addition of the mid-level point allows the efficient estimation of the coefficients of a second-order model.[15] Preliminary experiments in batch cultures showed that addition of ≥50 μM of the metal-sulphates was potent enough to induce changes in cell growth, lactate and glucose metabolism, and mAb production in the tested cell line. To test the limitations of metal supplements in fed-batch cultures, the metal supplement concentrations ranged from 0–200 μM.

The experimental design included 16 shake flask conditions (14 test conditions, including 3 center points) each supplemented with either zinc, copper, or combined zinc and copper at time of inoculation (0 h), at mid-exponential phase (60 h), or upon entering stationary phase (120 h), in addition to a control condition where no metals were supplemented. A 3D schematic of the experimental design space is displayed in Figure 1 and the list of final cell culture conditions is shown in Table 1. Metal sulphates diluted in cell culture medium were supplemented individually or combined at two levels – 100 and 200 μM – at the designated time setpoint.

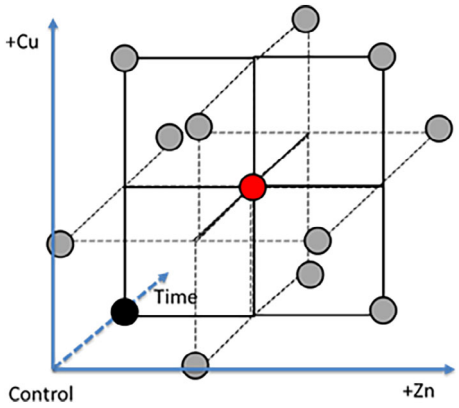

FIGURE 1 Schematic representation of the Box Behnkin DoE design employed to study time-wise trace metal supplementation impact on titer. 3D cubic and 3D axis representation of the Box–Behnken design where 3 factors are tested at three levels. ×1 is Zn supplementation at a range of 0–200 μM (3 levels, 100 μM is the mid-point), ×2 is Cu supplementation at a range of 0–200 μM (3 levels, 100 μM is the mid-point), and ×3 is the time of supplementation from 0 to 120 h (60 h being the mid-point).

2.4 | Antibody titer and glycan analysis

Cell culture samples were collected on day 8, spun down, supernatant collected, and stored at −20°C for titer measurements to be conducted at the end of the run. Protein titer was measured using an Agilent 1100 high-performance liquid chromatography (HPLC) system (Agilent, Santa Clara, CA) with a POROS® A 20 μM column (ThermoFisher Scientific, Waltham, MA). Antibody titer was calculated by fitting the elution peak areas to an IgG standard curve. Samples were measured in triplicate. Specific productivity was calculated from the following (Equation 1) on Days 7 and 8, the average of those two values was used for the statistical model data:

$$q_p = \frac{P_f}{\mathrm{IVCD}_{t_0}^{t_f}} \quad (1)$$

where q_p is the specific productivity, P_f is the is the concentration of antibody in mg/ml at time, t_f, and IVCD is the integral viable cell density ($\times 10^6$ cells-h/mL) over timeframe $t_f - t_0$.

Glycan structures were analyzed via high performance liquid chromatography (HPLC) using a method developed by Sha et al.[16] An Acquity UPLC BEH Glycan (HILIC Column) 1.7 μM – 2.1 mm × 150 mm HILIC Glycan Column was used with the buffer exchange method outlined in Table 2.

2.5 | Measuring relative levels of oxidative stress using DCFDA cellular ROS assay

Approximately 1 million cells were harvested from each shake flask on each day of ROS data collection using a 2′, 7′-dichlorodihydrofluorescein diacetate (DCFDA) Cellular ROS colorimetric assay kit (Life Technologies,

TABLE 1 Box-Behnken RSM experimental design conditions for time-wise zinc supplement screening on CHO-K1 cells and response variables. The experimental design includes 3 center points (CP) for reproducibility.

Exp No.	Description	Zn (μM)	Cu (μM)	Time	Note
1	0_Zn100	100		0	
2	0_Zn200Cu100	200	100	0	
3	0_Cu100	0	100	0	
4	0_Zn100Cu200	100	200	0	
5	60hr_Zn200	200	0	60	
6	60hr_Zn200Cu200	200	200	60	
7	120hr_Zn200Cu100	200	100	120	
8	60hr_Zn100Cu100	100	100	60	CP1
9	60hr_Zn100Cu100	100	100	60	CP2
10	60hr_Zn100Cu100	100	100	60	CP3
11	Control	0	0	0	Control
12	120hr_Cu100	0	100	120	
13	60hr_Cu200	0	200	60	
14	120hr_Zn100Cu200	100	200	120	
15	60hr_Zn100Cu200	100	200	60	
16	120hr_Zn100	100	0	120	

TABLE 2 Glycan HPLC buffer exchange method setup.

Time	% Acetonitrile	% Ammonia formate	Flow rate (mL/min)
0	75	25	0.3
62.5	40	60	0.3
70.0	0	100	0.1
76.0	0	100	0.1
82.0	75	25	0.2
87.0	75	25	0.3
120	75	25	0.3

Cat# C6827). Cells were spun down at 2000 rpm for 5 min and then washed with pre-warmed PBS. Centrifugation was repeated, wash buffer discarded, and cells were resuspended in 100 µL of DCFDA solution and incubated at 37°C for 30 min. Positive control samples were resuspended in 100 µM H_2O_2 prior to DCFDA treatment. Negative control samples (no DCFDA treatment) were measured to establish baseline fluorescence of the cells. Samples were assayed in triplicate. ROS release was measured every 2 min over 2 h using a BioTek Synergy HT (Agilent) microplate reader.

2.6 | Measuring cellular redox ratio (NAD+/NADH)

A total of 2 million cells were harvested from each shake flask and washed with cold PBS. Total NAD + NADH and NADH was quantified using a NAD/NADH Quantification Kit (Sigma Aldrich). Co-factor levels were measured at 450 nm absorbance using a BioTek Synergy HT (Agilent) microplate reader. Redox ratios were calculated using Equation 2 after subtracting blank values and normalizing by the cell count per assay (after sample dilutions, each assay well contained 2E+05 cells).

$$\text{Redox ratio} = \frac{\text{NADtotal} - \text{NADH}}{\text{NADH}} \quad (2)$$

2.7 | Enzyme activity assays (SOD1, LDH, and MDH)

SOD activity from cell lysates was assessed by measuring the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine in a convenient 96 well format (Cayman Chemical SOD Assay kit, cat# 706002). LDH and MDH enzyme activities were measured using colorimetric absorbance assays (Abcam, cat# ab102526, and ab183305, respectively). Each kit uses a standard curve of NADH to quantify the activity of LDH and/or MDH under kinetic mode. All enzyme activity assay samples were cell lysates prepared from 1 million cells, freeze-thawed twice followed by sonication for 20 min. Absorbance levels were measured on duplicate samples at 450 nm using a BioTek Synergy HT (Agilent) microplate reader.

2.8 | Statistical model analysis and validation run

After the cell culture dataset (including peak VCD, late-stage antibody titer, specific productivity, ROS activity, redox ratio, and enzyme activities) was collected and processed, a model was fitted using MODDE Pro 13 (Sartorius Data Analytics) using multiple linear regression (MLR) and checked for quality and outliers. The same dataset was used to generate a partial least squares (PLS) regression model, where cell culture parameter variables (metal concentration and time added) set as factors and late-stage cell culture performance data set as response variables. The optimizer function from the PLS model was then used to predict metal supplement concentration and feed times, which maximize mAb productivity while maintaining peak VCD within 10% of the control. The optimal setpoint (OPT) defined by the model was tested in a final cell culture run and along with a control (no metals added) and the highest titer (HT) condition from the initial DOE screening. Each validation condition was cultured in duplicate, including the control condition. Cell culture inoculation conditions were the same as performed for the initial optimization DOE except for higher initial glutamine concentration (9.25 g/L) in all conditions which triggered a feeding schedule that began 1 day earlier (day 3 instead of day 4).

3 | RESULTS

3.1 | Cell density and glucose profile impacted by early or high zinc levels, while copper reduced late-stage lactate levels

Figure 2 displays the viable cell density and viability data from the shake flask DOE. Zinc supplement during the growth phase (0–60 h) negatively impacted peak viable cell density, while addition of zinc at the end of the growth phase (120 h) had no impact on cell growth profile and improved harvest viability. In contrast, supplement of copper up to 200 µM at any point throughout the growth phase had no impact on cell density until the decline phase (days 7–9) where cultures with copper added maintained higher viability compared to the control culture (Figure 2b, e).

Addition of combined metals variably decreased CHO-K1 peak cell growth dependent on the zinc concentration and time added (Figure 2c, f). Here, cultures with supplements containing high levels of zinc (200 µM) and 100–200 µM copper at 0–60 h resulted in the highest reduction (~40%) in peak VCD. Combined metal-supplemented cultures with zinc added at the lower concentration (100 µM) at 0–60 h maintained peak VCD within 2%–18% standard deviation from the control condition. Supplement of combined zinc and copper after the exponential growth phase (120 h) had no impact on cell density after supplementation.

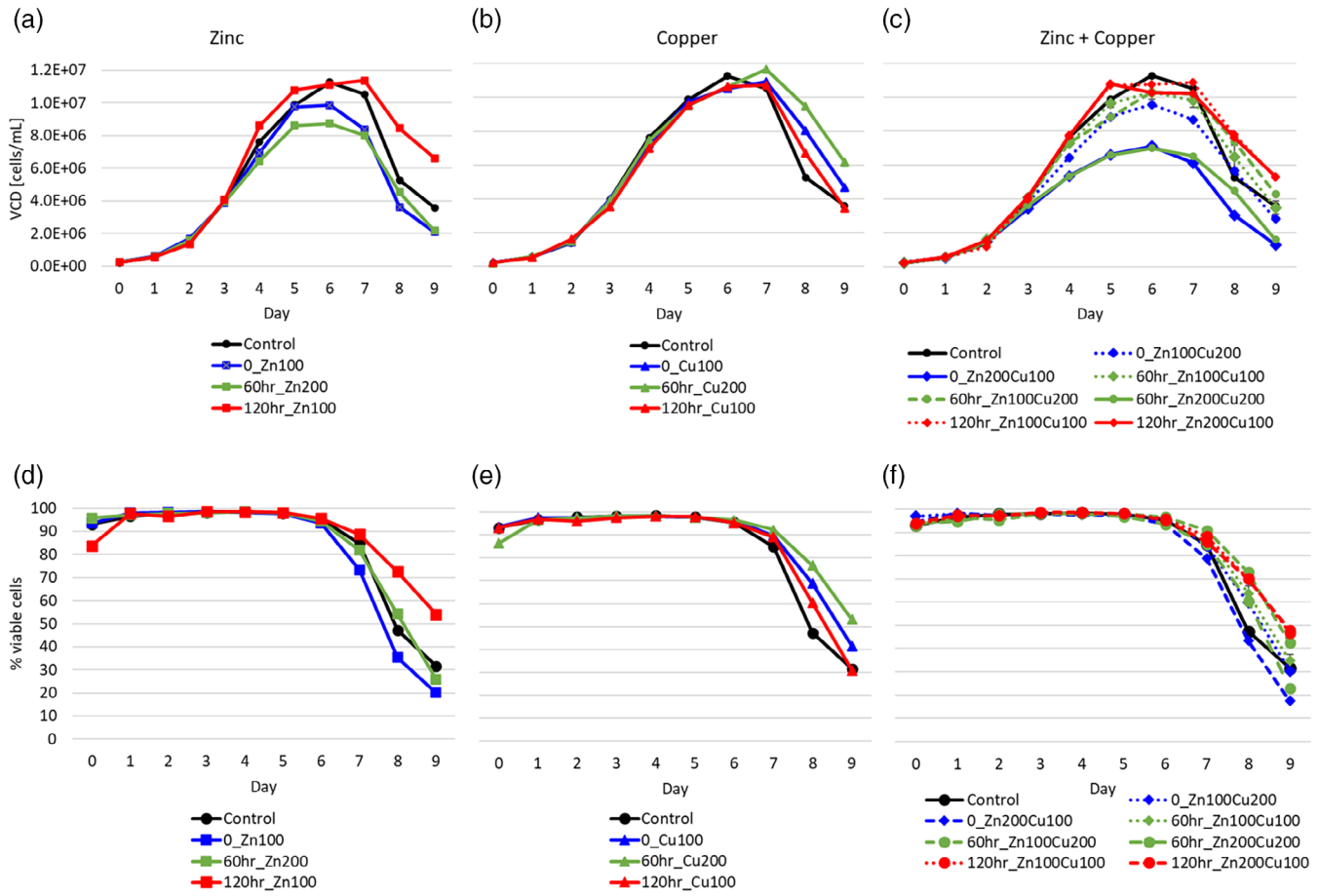

FIGURE 2 Cell growth and viability data from the DoE fed-batch runs. Viable cell density and percent viability profiles for zinc only (a,d), copper only (b,e), and zinc + copper (c,f) supplemented cultures. Solid black line = control condition, blue lines = metals added at 0 h, green lines = metals added at 60 h, red lines = single metals added at 120 h). Sample names are labeled time of supplement (h)_metal_concentration (μM).

Zinc and/or copper supplementation had no effect on culture pH, and glutamine levels throughout the culture duration (data not shown). Slight deviations occur in late-stage glucose profiles of cultures with zinc supplementation at 0 or 60 h (Figure 3a). In these cultures, higher glucose levels were maintained on the last 2 days of culture. Zinc supplement had essentially no impact lactate levels (Figure 3d), while copper slightly reduced lactate, levels at harvest (Figure 3b, e). Combined-metal supplemented cultures, with the exception of cultures with 200 μM zinc supplemented at 0–60 h, also exhibited reduced lactate levels at harvest while maintaining glucose profiles similar to the control condition (Figure 3c, f).

3.2 | Combined zinc and copper resulted in highest antibody titer and fewer galactosylated species

Averaged antibody titer values for day 8 samples harvested from each of the DOE shake flasks is shown in Figure 4, where changes in antibody titer were deemed statistically significant based on p-values. Cultures under early zinc supplementation (0–60 h) yielded higher antibody levels by the end of cell culture. Delayed supplement of 200 μM zinc at 60 h increased titer by 49% compared to a 22% increase when 100 μM zinc is added at inoculation. Copper supplement increased titer most when added at inoculation, yielding approximately 50% more antibody compared to the control condition. Interestingly, even further titer enhancement occurred when coupling 100 μM zinc with 200 μM copper (a 1:2 ratio of zinc and copper) supplementation at 0 h compared to single metal addition. In this condition, an average 77% higher titer was observed compared to the control condition. Other combined metal conditions resulted in ≥40% higher antibody concentration, even when added at 120 h.

To assess the effects of single versus combined zinc and copper treatments on product quality, glycan distributions for the recombinant IgG product accumulated by day 7 were measured (Figure 5). Changes in galactosylation were deemed statistically significant based on p-values. HPLC glycoform analysis of day 7 samples showed apparent reduction in galactosylated species across all metal-supplemented cultures. Addition of 100–200 μM zinc at 0 or 60 h led to ~8% decrease in galactosylated species, while later supplementation of zinc at 120 h showed less reduction (only ~3% decrease). Addition of

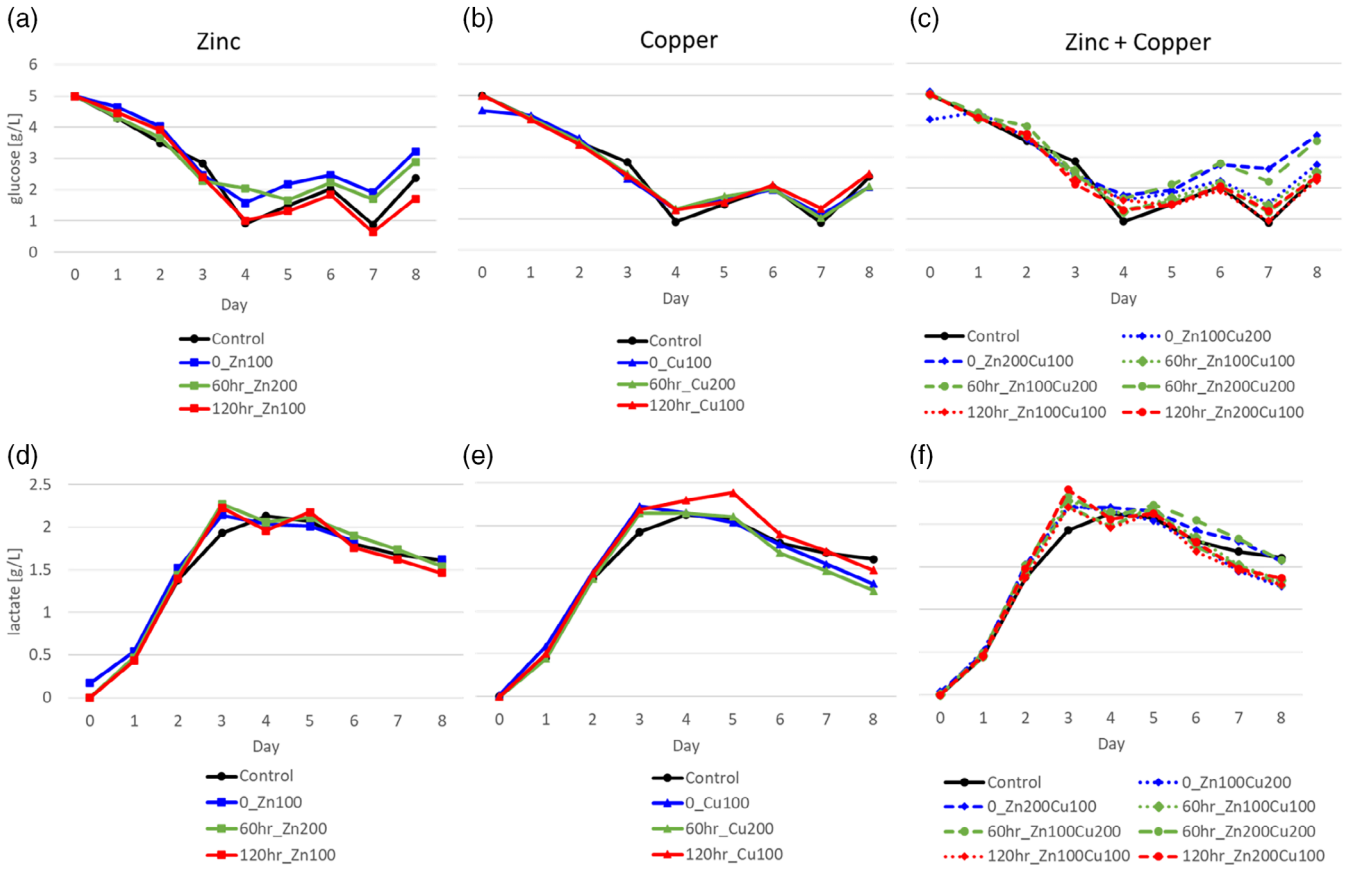

FIGURE 3 Glucose and lactate profiles for zinc only (a,d), copper only (b,e), and zinc + copper supplemented cultures (c,f). Solid black line = control condition, blue lines = metals added at 0 h, green lines = metals added at 60 h, red lines = single metals added at 120 h. Sample names are labeled time of supplement (h)_metal concentration (μM).

copper sulfate alone had a similar effect on galactosylation with ~5% reduction in galactosylated species at all time points. Combined metal supplementation resulted in the highest reduction in galactosylated species ranging from ~3% to 15%. When 200 μM of zinc combined with 100–200 μM of copper are supplied at 0 or 60 h, a ~ 15% reduction in galactosylated species is observed. The condition with the most significant titer increase (100 μM zinc and 200 μM copper supplemented at 0 h), resulted in a 12% reduction in galactosylated IgG. Delayed addition of zinc and copper at 120 hours had less impact than early addition, with only 3%–5% increase in G0F species.

3.3 | Combined zinc and copper supplementation reduced oxidative stress and cellular redox ratio

The qualitative change in ROS activities from day 0 to day 6 across culture conditions are displayed in Figure 6. Changes in ROS activity were deemed statistically significant based on p-values. The control condition (no metals added) resulted in the highest ROS activity level by day 6. All conditions where zinc and copper were supplemented, at every time point, resulted in various levels of reduced ROS activity (10%–50%). An incremental increase in ROS activity was observed as either combined or individual metals were added later during the growth phase. The largest reduction in ROS activity (50%) was measured from cultures with early copper addition with and without zinc. Overall, combined zinc and copper supplement at every timepoint consistently yielded lower ROS activity levels compared to single metal supplemented cultures.

Quantitative analysis of the relative concentrations of two critical enzyme cofactors, NAD+ and NADH, revealed various alterations in cellular redox ratio upon single and combined metal addition (Figure 7). By day 7, the control condition (no metals added) had a NAD+/NADH ratio of 10:1, meaning there were approximately 10× more NAD+ available in the cells than NADH. Supplement of zinc at any time point resulted in a ≥ 50% drop in NAD+/NADH by day 7. Copper supplementation reduced NAD+/NADH ratio similar to zinc when added at the start of culture (0 h). However, when copper was supplemented during late-stage growth (120 h), a sudden increase in NAD+ availability was observed compared to when copper was supplemented early (0 h). The majority of combined metal supplemented cultures resulted in decreased NAD+/NADH ratio, even when added during the onset of the stationary phase, the exception being conditions with high zinc (200 μM) added early (0–60 h).

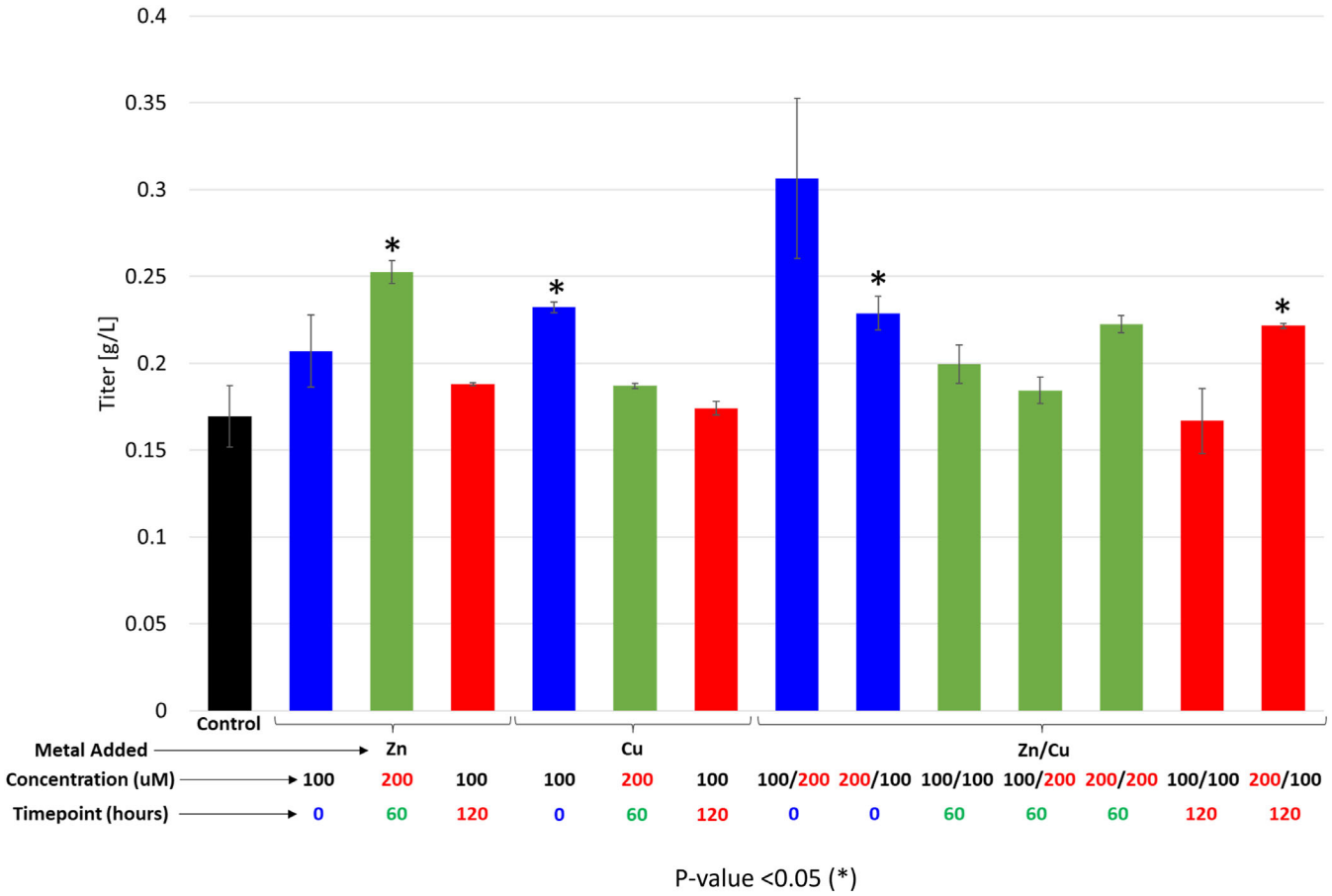

FIGURE 4 IgG titer values for zinc, copper, and zinc+copper supplemented cultures on day 8. Solid black bar = control condition (no metals added), blue bars = conditions where metals were added at 0 h, green bars = conditions where metals were added at 60 h, red bars = conditions where metals were added at 120 h. Labeling at the bottom of the chart indicates the metal added, concentration (μM), and timepoint of supplementation. Combined addition of 100 μM zinc and 200 μM copper at 0 h resulted in the highest IgG titer. *P*-value <0.05 (*).

3.4 | Zinc supplement increased LDH activity, with further enhancement when added with copper

Enzyme activity data revealed some interesting trends across the three enzymes assayed. For single metal addition, LDH demonstrated higher activity with zinc or copper addition, with variability depending on the time and concentration added (Figure 8). Higher zinc concentration seems to drive higher LDH activity, as addition of 200 μM zinc alone at 60 h resulted in activity close to the levels seen with combined metal addition. Copper supplementation resulted in similar elevated LDH activities when added at 100 μM at 0 or 120 h, however the highest copper concentration (200 μM) yielded no change in late-stage LDH activity. Combined metal supplement cultures consistently resulted in higher LDH activities compared to most single metal added cultures, exhibiting a combinatorial effect of zinc and copper on LDH. The highest increase in LDH activity (~ 350 mU) occurred under combined zinc and copper supplement the highest dosages (200 μM of each).

Unlike LDH, SOD1 and MDH activities were essentially maintained within 10% [mU] in the majority of the DOE conditions (Figure 9). Cultures supplemented with either zinc or copper at the start of culture (0 h) led to slightly decreased SOD1 enzyme activity by day 7 compared to the control condition. Similarly, combined 200 μM zinc and 100 μM copper at 120 h (day 5) also led to slightly reduced SOD1 activity by day 7. However, when both metals were added at either 1:2 or 2:1 ratio of Zn:Cu concentration during early or mid-exponential growth phase (0–60 h), SOD1 activity was relatively maintained. MDH activity remained within 10% [mU] across most conditions, with exception for the condition with the highest concentrations of both metals added at 60 h. Here, the specific MDH enzyme activity drastically decreases, most likely an indication of the cytotoxicity of the metals added at those concentrations.

3.5 | Response surface optimization regression analysis and predicted setpoint for enhanced specific productivity

The summary of fit statistics for the MLR model suggested that the variation in specific productivity and peak VCD are well explained by the model with good prediction capability (R2 = 0.97, Q2 = 0.64 and R2 = 0.95, Q2 = 0.41, respectively). A PLS model was constructed

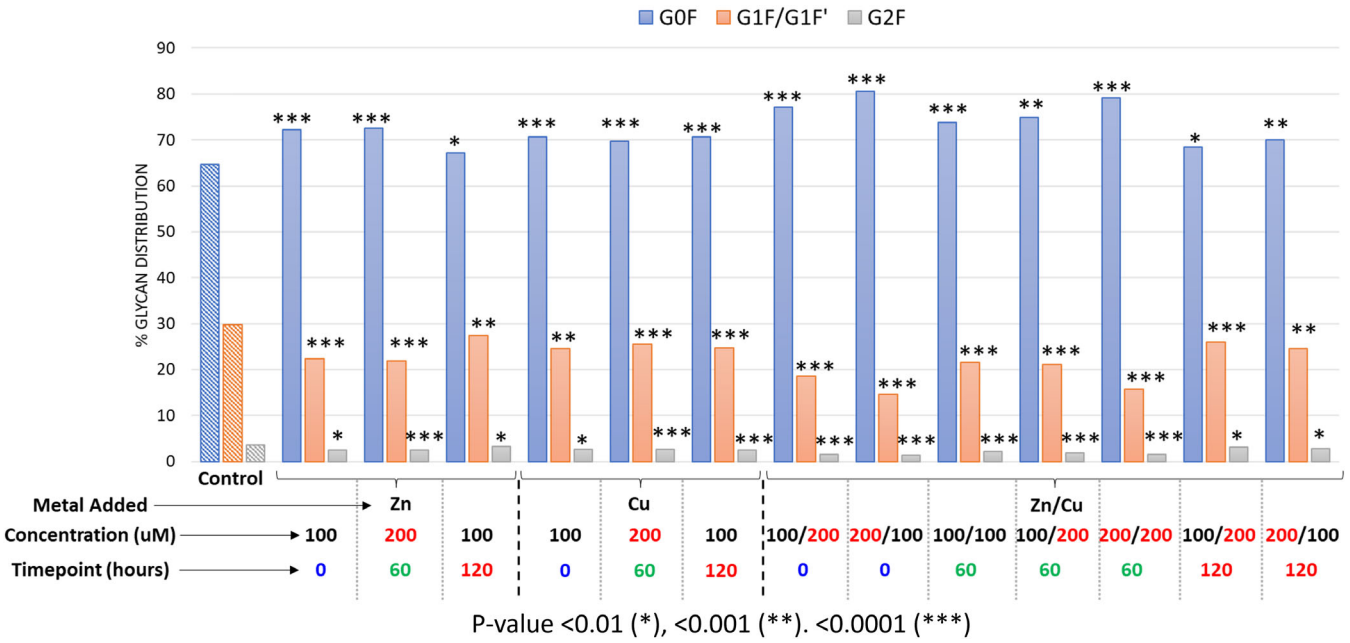

FIGURE 5 Day 7 glycan distribution of G0F, G1F/G1F', and G2F. Labeling at the bottom of the chart indicates the metal added, concentration (μM), and timepoint of supplementation. Combined supplement of zinc and copper show the greatest reduction in glycosylated IgG product. *P*-value <0.01 (*), <0.001 (**). <0.0001 (***).

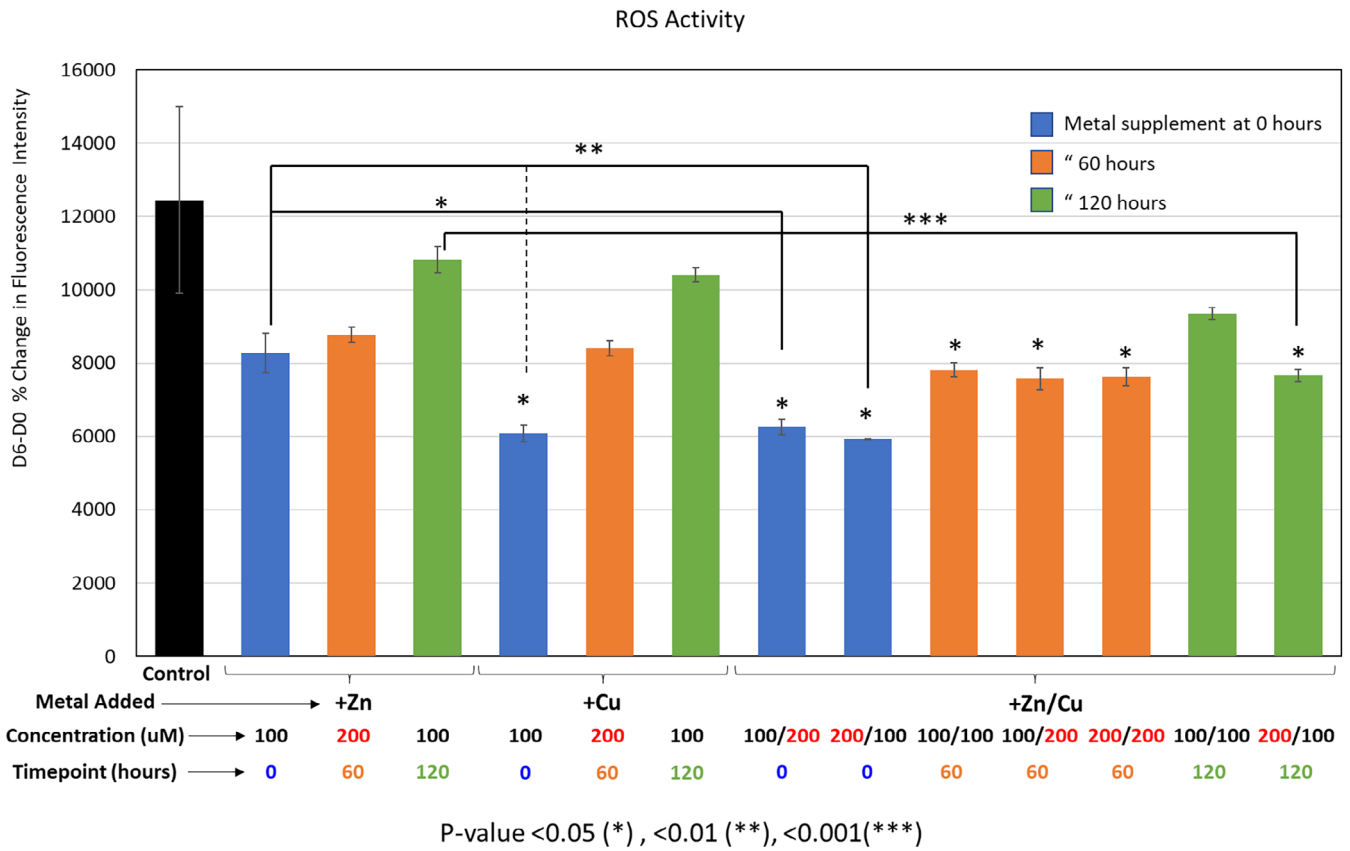

FIGURE 6 Percent change in ROS activity (fluorescence signal) from day 0 to 6 for each DOE condition. ROS activity was measured using a DCFDA fluorescence assay. Solid black bar = control condition (no metals added), blue bars = conditions where metals were added at 0 h, orange bars = conditions where metals were added at 60 h, green bar = conditions where metals were added at 120 h.

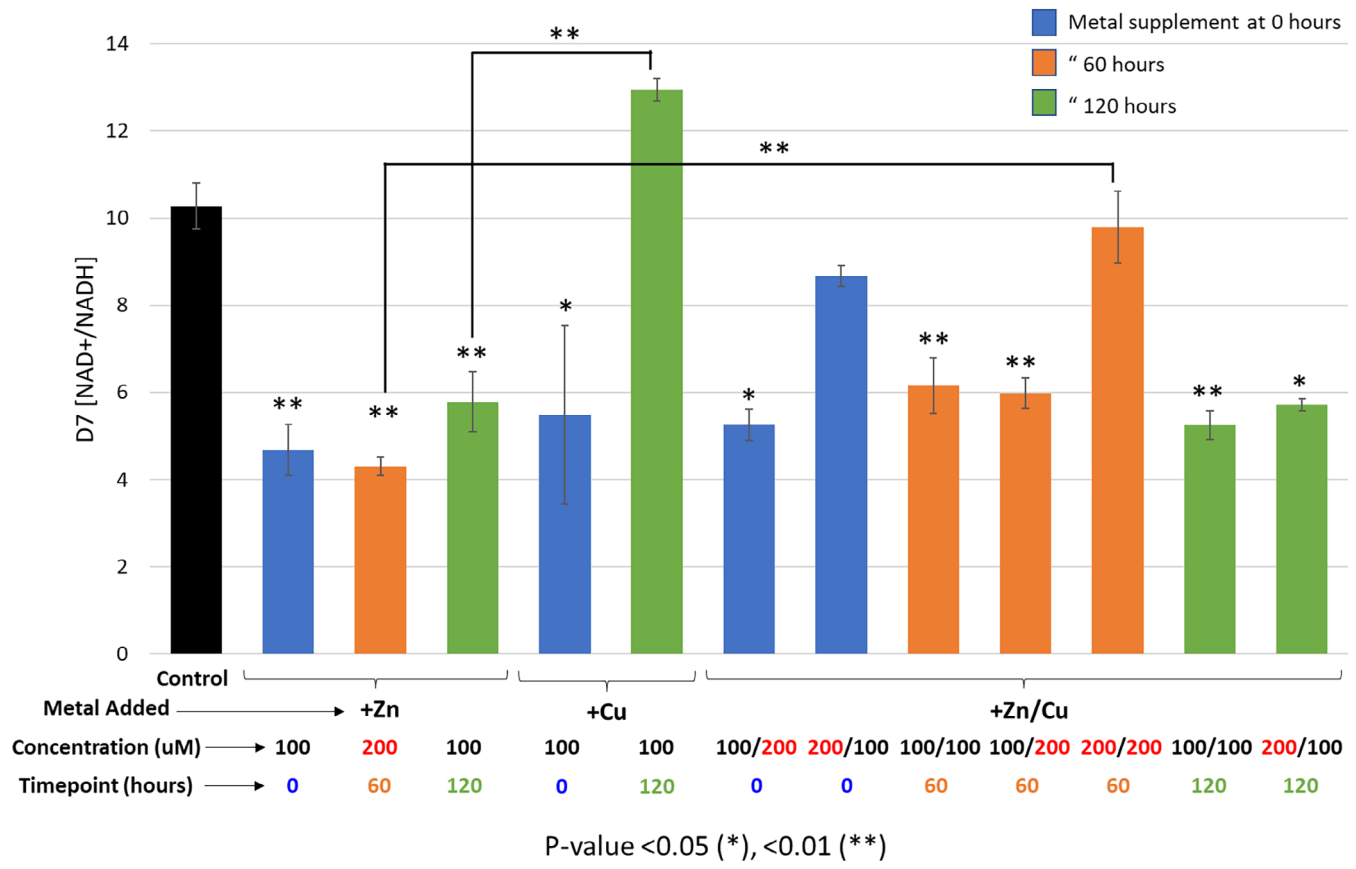

FIGURE 7 Day 7 cellular redox ratio (NAD+/NADH) data from DOE conditions. Solid black bar = control condition (no metals added), blue bars = conditions where metals were added at 0 h, orange bars = conditions where metals were added at 60 h, green bar = conditions where metals were added at 120 h.

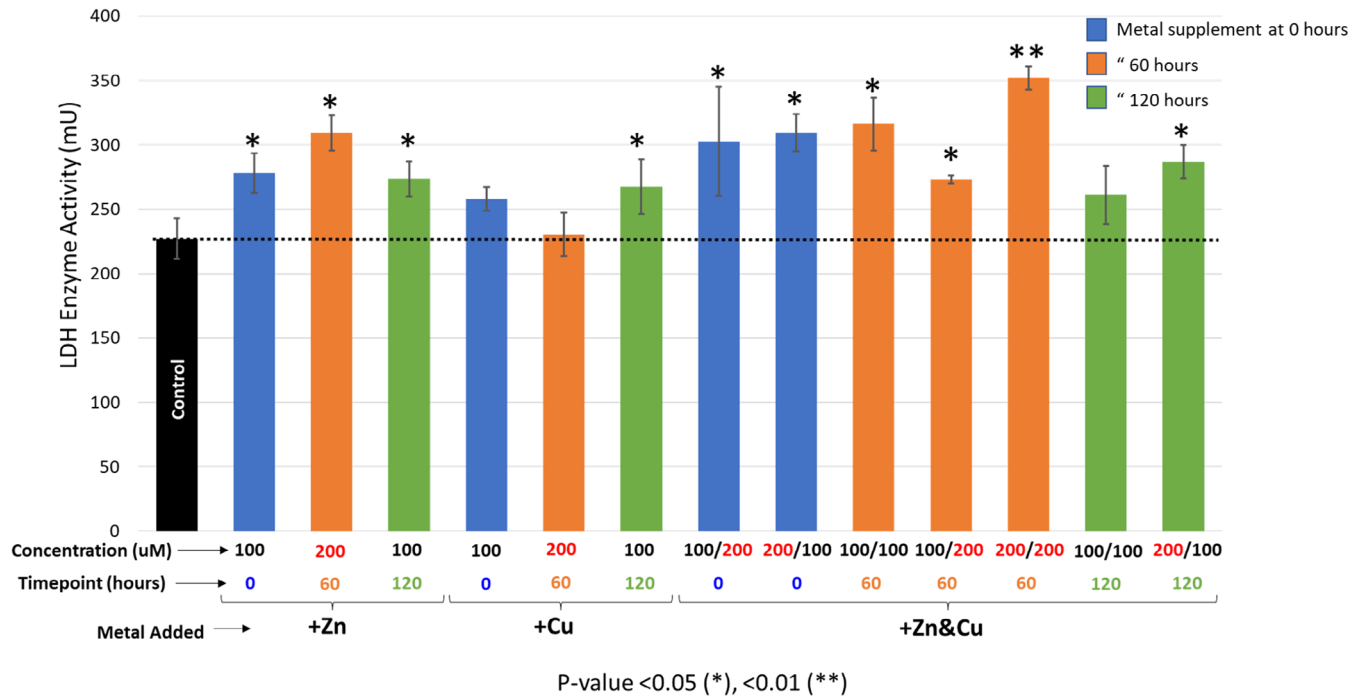

FIGURE 8 Day 7 enzyme activity of LDH. Solid black bar = control condition (no metals added), blue bars = conditions where metals were added at 0 h, orange bars = conditions where metals were added at 60 h, green bar = conditions where metals were added at 120 h.

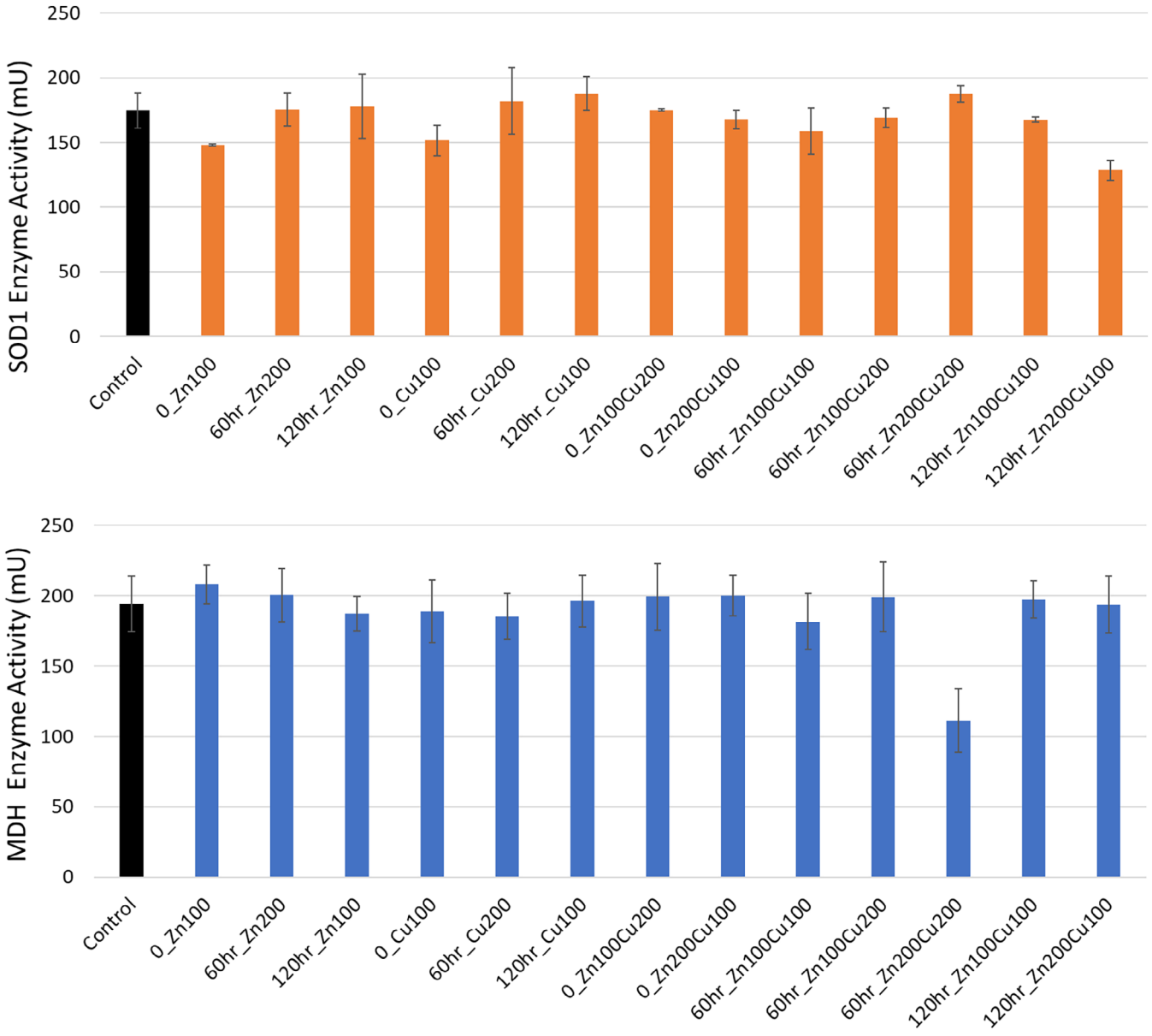

FIGURE 9 Day 7 SOD1 (top chart) and MDH (bottom chart) enzyme activity. Solid black bar = control condition (no metals added). Numbers before metal abbreviations indicate the hour of metal supplementation. Numbers after metal abbreviations indicate the concentration in μM of each metal sulphate (either zinc or copper, or zinc and copper) supplemented.

for each response variable, and the validity and reproducibility of the models were assessed based on the summary of fit data shown in Figure 10. Also summarized in Figure 10 are the percent of the variations of the response explained by the model (R2), and the percent of response variation that the model can predict (Q2). Out of the 7, PLS models generated, specific productivity and peak VCD had the highest R2 and Q2 values at ≥0.88 and ≥0.52, respectively. Harvest titer and LDH enzyme activity exhibited high R2 values of 0.75 each, and high model validity of 0.65 and 0.95, respectively. However, it should be noted that both of these response variables resulted in below acceptable Q2 values of 0.18 and 0.24, indicating the model's poor predictive capability. ROS Activity, Redox Ratio, SOD1 Enzyme Activity, and MDH activity models had extremely poor (negative) Q2 values, yet ROS and enzyme activity models exhibited high model validity and acceptable reproducibility. As the redox ratio model was deemed invalid, and the MDH activities exhibited no significant correlation to the factors based on the regression coefficients (data not shown), these two response variables were excluded from the subsequent model analysis steps. Variable Importance in the Projection (VIP) Plots summarize the importance of the model terms in explaining X and correlating to Y. Values above 1 indicate important terms for each model. Significant factors were then narrowed down through analysis of the regression coefficient plots, where factor significance is weighted by the direction (positive or negative) and size of the confidence interval. Here, titer & specific productivity (qP) were strongly correlated based on their coefficient values and directions, which makes sense since

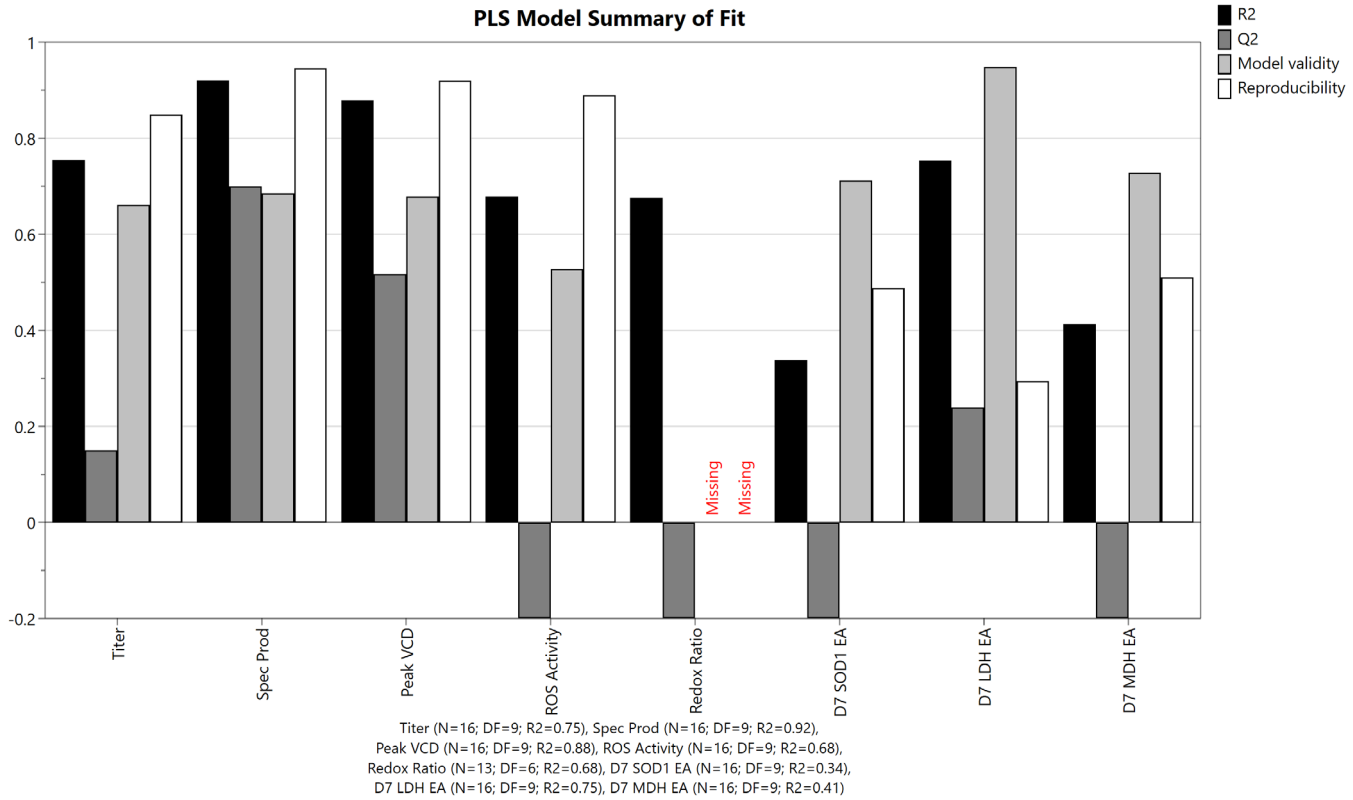

FIGURE 10 Summary of fit plot showing RSM model fit (R2), predictability (Q2), model validity, and reproducibility. (N: sample size; DF: Degrees of Freedom).

specific productivity is calculated using titer values. Shown in Figures 11 and 12, the significant factors that influence specific productivity and peak VCD were zinc concentration (positive/negative for specific productivity and peak VCD, respectively), time added (negative/positive), and the interaction between zinc and time added (Z*t) (negative/positive) based on a 0.7 VIP threshold and confidence intervals which do not span the origin. ROS activity correlated with copper concentration (negative) and time added (positive), while LDH enzyme activities correlated with zinc concentration (positive), and none of the terms correlated significantly with SOD1 enzyme activity.

The optimizer function was used to identify an optimal setpoint based on the model regression data for both enhancing specific productivity while maintaining peak VCD within a target range (10%). The model objective also desired minimized ROS activity and redox ratio, as well as maximized LDH activity and maintained SOD1 and MDH activity. The "optimized" setpoint with the lowest log(D) and probability of failure was selected the optimizer function, which was supplement of 80.12 μM zinc and 199.98 μM copper at 0.05 h.

3.6 | Validation run confirmed regression model's predictive capabilities

A validation run was performed to confirm the effects of combined metal condition on antibody production and test the alternative "optimized" setpoint designated by the optimizer function of the regression model. Viable cell density and cell viability profiles from the validation experiment are shown in Figure 13 (top) and showed that no significant changes in peak VCD occurred due to combined metal supplementation at these concentrations. In fact, due to the higher glutamine supplement and modified feeding schedule, all conditions resulted in a higher peak VCD range and slightly higher titer compared to the initial DOE. Yet, the test conditions with combined zinc and copper supplement still yielded over 20% higher titer due to enhanced specific productivity (>40% higher) compared to the control condition (no metals added), as shown in Figure 13 (bottom). Although decreasing the zinc concentration did slightly improve late-stage viability, antibody titer and specific productivity were not enhanced further in the predicted optimal (OPT) condition compared to the original high titer (HT) condition from the DOE. The validation run provided evidence that combined zinc and copper addition enhanced the specific productivity in the investigated CHO-K1 cell line but the results from the initial screening were not reproduced likely due various reasons addressed in the discussion.

4 | DISCUSSION

This study identified some interesting correlations between individual zinc and copper versus combined metal addition and the subsequent bioprocess outcomes. Although several bioprocess outputs were

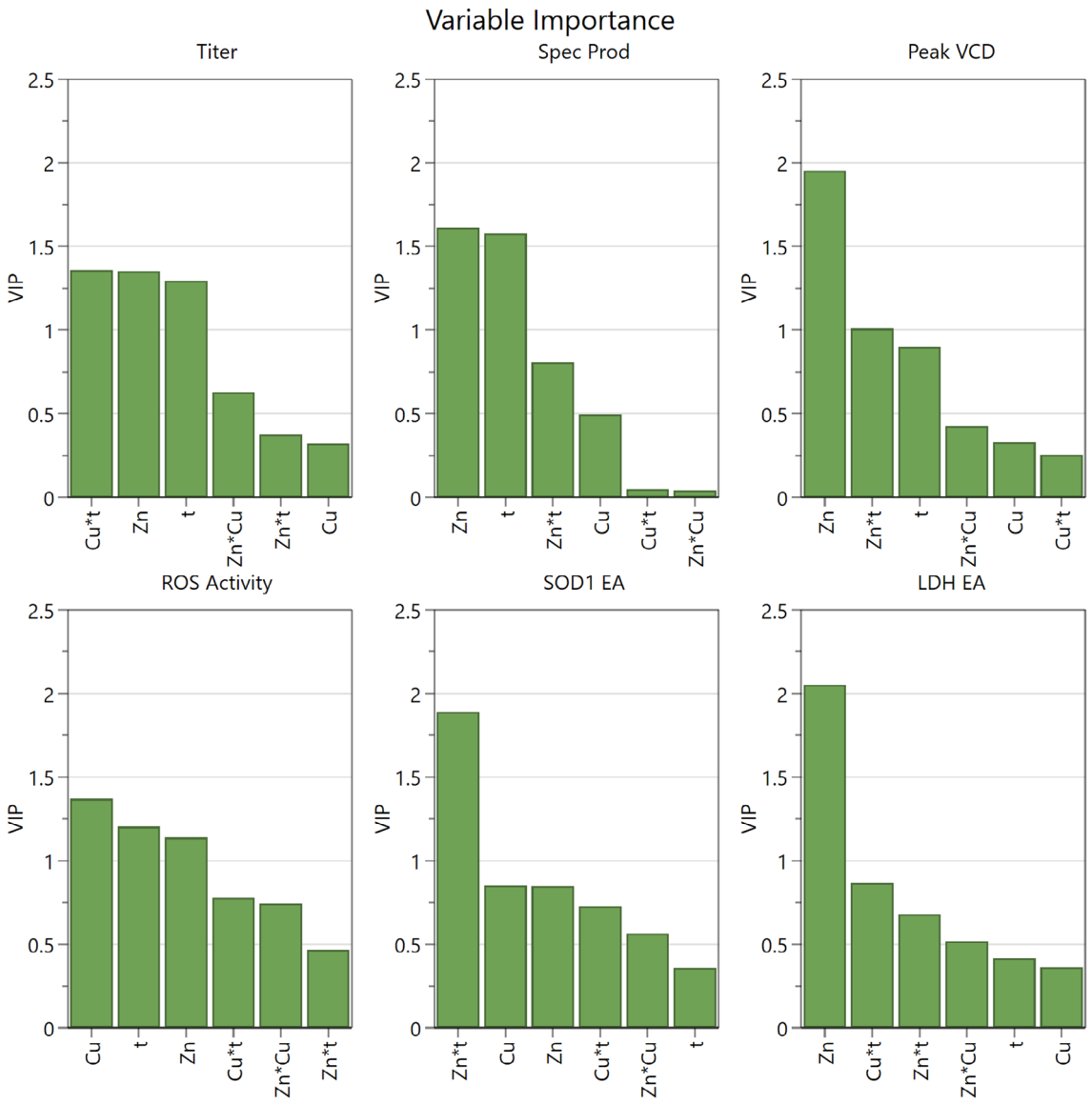

FIGURE 11 Variable Importance in the projection (VIP) plots for the response variables: titer, specific productivity, peak viable cell density (Peak VCD), ROS activity, SOD1 enzyme activity (SOD1 EA), and LDH enzyme activity (LDH EA). VIP Plots summarize the importance of the model terms in explaining X and correlating to Y. Values above 1 indicate important terms for each model.

measured, the regression model identified that peak VCD and specific productivity (and thus titer) are the response variables that are most significantly impacted by zinc, copper, and time of supplementation and are best fitted by a regression model. Additionally, ROS activity and LDH activity had significant model validity and explained variability based on the statistical model analysis. Redox ratio, SOD1 enzyme activity, and MDH enzyme activity were poorly modeled by the dataset, and therefore effects of zinc and copper on these factors will not be included in the discussion. Focus will remain on statistically significant and well modeled factors including peak VCD (or cell growth), specific productivity/titer, ROS activity, and LDH activity in response to time wise addition of zinc and copper.

Cell performance data revealed that the CHO cell line is more sensitive to zinc compared to copper supplementation at the tested concentrations. However, pairing zinc with copper addition can help alleviate negative effects that zinc has growth and thus improve antibody production further *if* balanced properly with copper. The optimal ratio of these metals to supplement ultimately depends on the cell line, time of addition, and the desired process outcome. For example, early combined addition of metals improves titer the most when supplemented at a 1:2 ratio, however the glycosylation profiles change the most. On the other hand, delayed zinc supplementation at 120 h did not impact the cell density and glycosylation to the extent seen with early supplementation, and still antibody titer improved when the ratio of zinc to copper added was 2:1. The PLS model for peak VCD confirmed negative and positive correlations with zinc and time added, respectively, while specific productivity/titer have the same significant terms but with opposite correlations. This is unsurprising due to the extremely high titer observed in the early

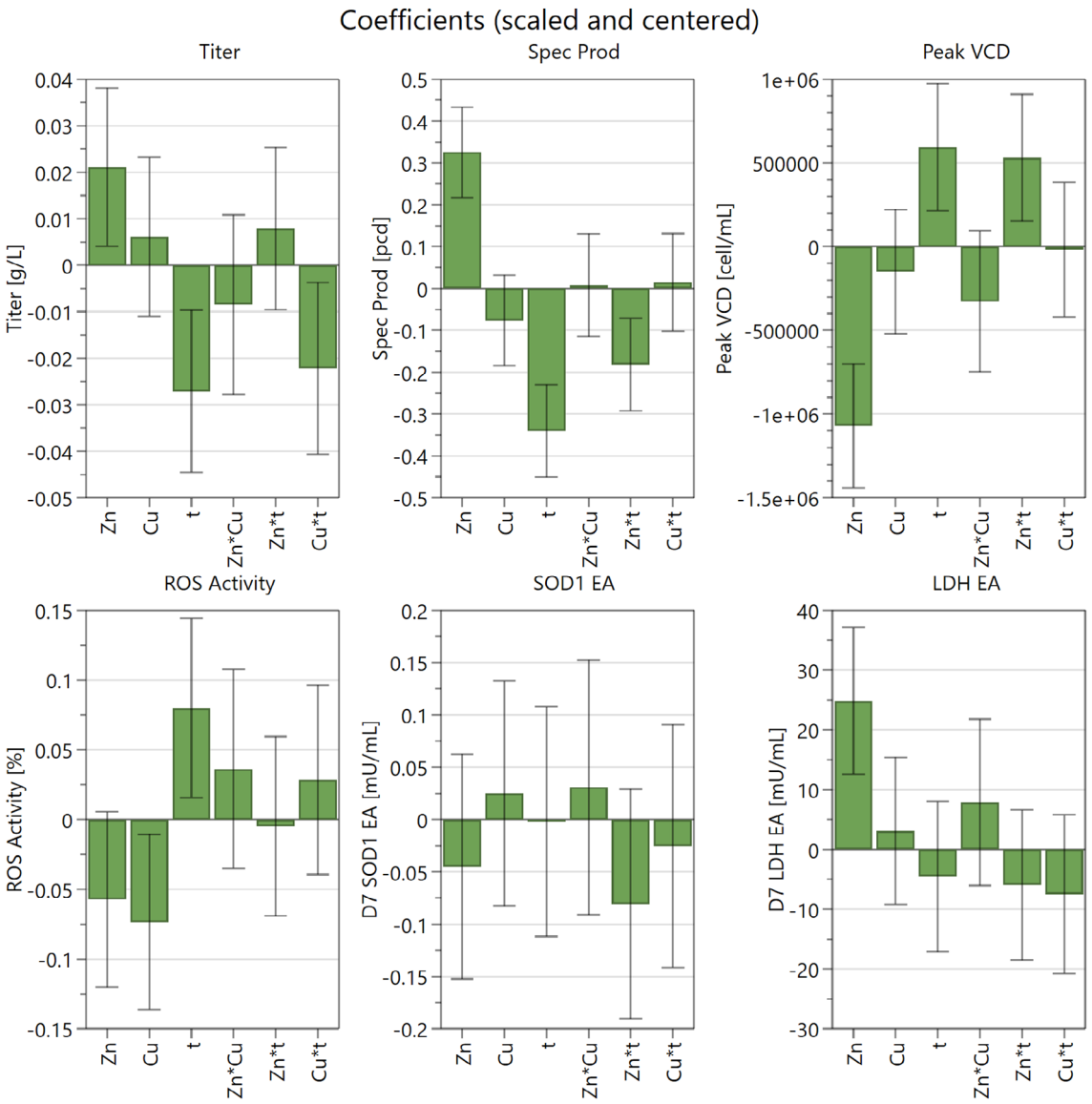

FIGURE 12 Regression coefficient plots for each of the response variables. A term coefficient is significant when the confidence interval does not cross zero.

combined metal and zinc supplemented condition, which is likely influencing the model output. The model also does not consider the combinatorial effects of zinc and copper addition on glycosylation, not to mention other product quality attributes. Therefore, improvements to the model could made by incorporating replicate time course data and running additional conditions with higher concentrations of copper and lower concentrations of zinc. The validation run confirmed the lack of model robustness and predictive capability, as the optimal condition did not achieve titer anywhere close to the initial high concentration observed during the DOE screening. However, it should also be noted that the differences in cell culture performance observed in the validation experiment could be due to other changes to cell culture parameters such as the higher glutamine levels and slight change in feeding schedule.

The reduction in cell growth observed upon early and high zinc supplement is likely an effect of reduced glucose consumption via the glycolysis pathway. This effect makes sense, as glucose metabolism does depend on zinc availability, as shown in previous zinc deficiency studies.[17,18] Excess zinc availability or "zinc overload" during early cell growth can slow down the rate of glycolysis significantly by either binding directly to GAPDH or affecting the activity of zinc-finger proteins and/or LDH which would hinder cell growth.[19] In this study, early zinc supplement may be beneficial to CHO cells because it affords them reduced dependence on glycolysis and enhanced oxidative pathway activation as an additional source of energy.[20] In other words, cells were able to utilize more energy resources during late-stage culture toward producing antibody instead of oxidative damage control. This theory is supported by the reduced cellular ROS activity

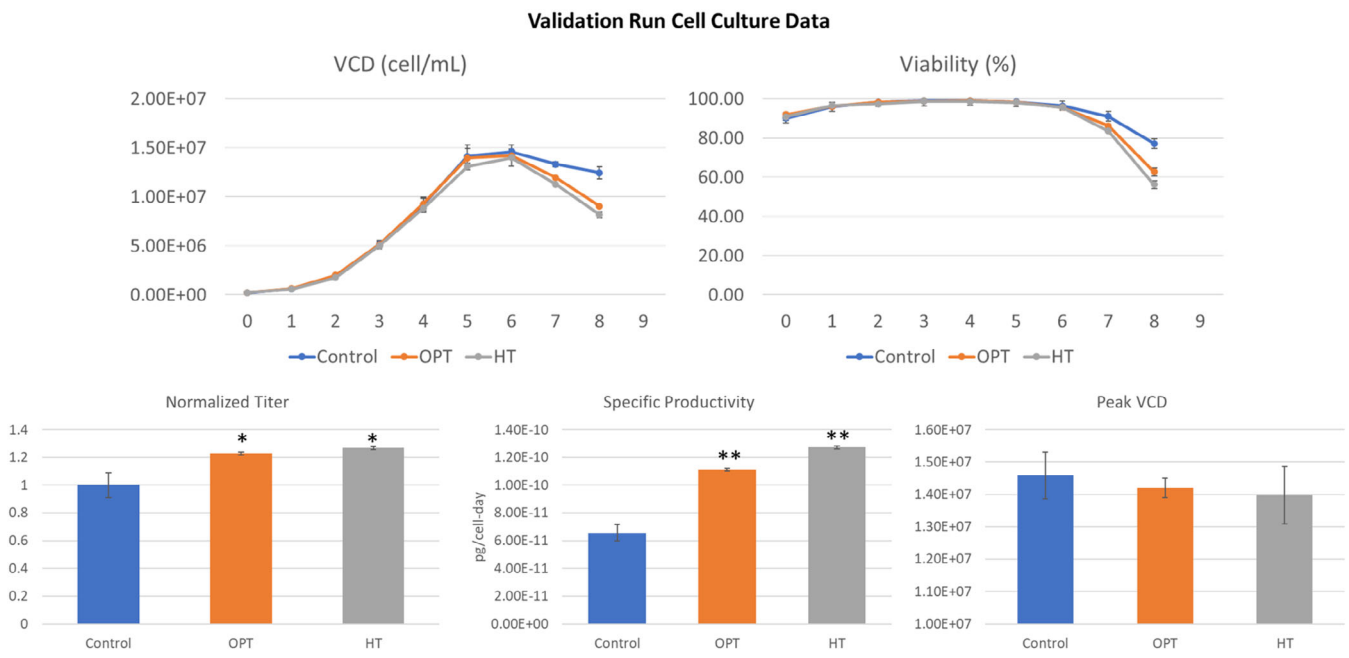

FIGURE 13 (Top) Growth and viability profiles from the validation run. Control = no metals added, OPT = optimal setpoint defined by the regression model (80 μM Zn, 200 μM Cu), HT = high titer condition from the initial DOE (100 μM Zn, 200 μM Cu). (Bottom) Day 8 normalized titer values for each validation test condition (*p-value <0.001), day 8 specific productivity values (**p-value <0.0001), and day 6 peak VCD values for each test condition.

and higher antibody levels observed in zinc supplemented cultures despite the cell density being impacted.

The reduced ROS activity observed in all metal supplemented cultures indicated that the cells were in a reduced oxidative stress state. The PLS model generated from the cell culture data indicated a significant correlation (based on coefficients plot) between copper concentration and time added to day 6 ROS activity. Reduced oxidative stress due to either zinc or copper alone has been previously reported,[8,21] however raw data from this DOE revealed that combined zinc and copper supplementation provides even further reduction in late-stage ROS activity than early zinc supplement alone, and early copper supplement reduces ROS to a greater extent than zinc. These results may be cell line specific, however, and will need to be validated across other cell lines.

The insignificant variability in SOD1 enzyme activities under metal supplementation support the notion that reduction in ROS activity observed on day 6 was not a direct result of metals impacting late-stage SOD1 enzyme function. Instead, cells could be utilizing alternative mechanisms of reducing ROS species upon metal supplementation, such as zinc counterbalancing the affinity of copper and other metals to oxidize free thiol residues and induce Fenton chemistry,[22] or copper-induced reduction of ROS species via cytochrome oxidase.[23] Although early-stage changes in SOD1 activity due to combined zinc and copper metal supplement is a possible contributor to late stage reduced ROS accumulation, by day 6 the SOD1 activity had stabilized. It is also noted that only cytosolic Zn/Cu SOD1 was measured here when there are two other isoforms of SOD1, which exist in other locations (Mn-SOD (SOD2), and Fe-SOD) which also play a role in oxidative stress within the mitochondria. Depletion of ROS activity could also indicate an imbalance of ROS between the cytosol and mitochondria, as changes in mitochondrial ROS activity were not quantified here. Imbalance between the mitochondria and cytosol ROS could lead to build up or reduction of NAD+ concentration in the cytosol by the stationary phase, as seen in majority of the metal supplemented cultures. Additionally, it is well noted that the shake flasks used for this work are not suitable for maintaining DO above 0%–10%, and therefore this study serves as a proof of concept to how metals can influence ROS in CHO cells in a low-DO environment. It is expected, however, that the results could vary substantially if the experiment were mimicked in a pH/DO-controlled bioreactor.

As shown by the raw data and PLS model, late-stage LDH enzyme activity demonstrates a strong correlation with zinc concentration in the cell culture media, with even further enhanced enzyme activity when supplemented in combination with copper. Earlier addition (at 0–60 h) increased the late-stage activity to a further extent than late addition (120 h), implying that zinc's effect of enhancing LDH activity could be gradual over the course of days and would only be observed in extended culture. To confirm this, time course LDH activity data would be beneficial to examine the changes in the enzyme's activity over time. The link between LDH activity and zinc has been investigated previously, however, the opposite trend was observed where excess intracellular zinc availability reduced the activity or increased efflux of intracellular LDH.[24] One explanation of these confounding results is the zinc delivery system and the type of assay used to measure LDH activity, as zinc oxide (ZnO) nanoparticles and in vitro toxicological assays were used to deliver zinc and assess LDH

levels, respectively. Additionally, the authors explain that the LDH levels measured in in vitro toxicological tests of ZnO nanoparticles cannot be used directly for interpretation, as artifacts in toxicological assays can lead to erroneous estimation of particles toxicity.

Zinc's antioxidant capability is likely impacting the LDH activity via regulation of the cells' redox ratio, or the relative intracellular levels of NAD+ to NADH. Cellular redox ratio, here measured by NAD+/NADH, is a regulator of cellular metabolism. A high redox ratio would indicate a higher rate of various mechanisms throughout the cell, such as the consumption of lactate or glucose, the conversion of pyruvate to acetyl CoA, the conversion of malate to oxaloacetate, and other steps of the TCA cycle – all of which depend on NAD+ availability and support cell growth and productivity. Typically, the cytosolic pool of NAD+/NADH differs across various tissues but is estimated to range between 0.1 and 10 (3–10 in mammals) and slight changes in the baseline ratios of cytosolic NAD+/NADH can function as a metabolic regulator.[25] The control condition (no metals added) revealed a baseline redox ratio of ~10:1 in this CHO-K1 cell line on day 7. In this case, a sustained NAD+/NADH redox ratio in the cytosol in metal supplemented conditions would indicate preserved energy metabolism.[26] However, the majority of metal-spiked conditions resulted in ≥40% drop in NAD+/NADH ratios compared to the control condition, which is indicative of reduced energy metabolism flux. Although there is still much more NAD+ than NADH available to drive co-factor dependent reactions, the rates of those same reactions are most likely altered compared to the control condition. Here, reduced availability of NAD+ in the cytosol could lead to decreased glycolytic flux via GAPDH, yet still result in increased lactate consumption via generation of pyruvate. Furthermore, zinc supplementation may somehow be impacting glycerol-3 phosphate biosynthesis, which was recently identified as an endogenous cytosolic NAD+ regeneration pathway.[27] Accumulation of glycerol is another indication of the cell attempting to restore redox balance. Glycerol is produced when dihydroxyacetone phosphate (DHAP) is converted to glycerol-3phosphate alongside the oxidation of NADH. When glycerol accumulates in CHO cells, glycerol 3-phosphate was reported to be upregulated upon mAb production.[28] This is another reaction the cells may be using to restore cytosolic NAD+/NADH so that higher LDH activity can be sustained. Previous research has shown that stable transfection of human cytosolic glycerol-3-phosphate dehydrogenase led to 8-10-fold decrease in NAD+/NADH ratio in CHO cells and ultimately resistance to oxidative stress.[29] However, to date, metal antioxidants have not been used to modulate glycerol-3 phosphate biosynthesis or NAD+/NADH in CHO cells, and confirmation of decreased expression or enzyme activity under zinc and/or copper supplement should be confirmed. In our study, the PLS model contained insufficient redox ratio data to accurately make significant correlations among redox ratio and the various response factors analyzed, therefore additional data on redox ratios throughout the culture duration would be useful to generate a valid model.

The effects of zinc and copper supplementation on CHO cell productivity have been evaluated, as previous research has shown zinc supplementation of 25 mg/L increased IgG titer up to 2.6 fold, and no significant change in titer upon copper supplementation up to 20 mg/L.[9] In the present study, a different cell line was used and up to 2× higher concentrations of zinc and copper were supplemented in a timewise fashion. Here, delayed zinc supplement at 60 h resulted in further titer increase (and significant boost in cellular productivity) compared to early addition, while early copper addition also increased titer. This study reveals for the first time that delayed zinc supplement or early copper may be ideal for enhancing late-stage productivity with least alteration to the glycosylation profile.

Supplement of copper at the start of culture led to higher final antibody titer compared to delayed supplement. This outcome may be due to the copper-depleted basal media used in this study, and therefore addition of copper at 0 h ultimately improved overall cell health and productivity. This notion is also supported by the statistical model, as the only factor that copper correlated with significantly was ROS activity, and those cultures exhibited the lowest ROS activity. A study using a cell line adapted to a media with an acceptable baseline concentration of copper had been investigated previously, specifically to understand the effect of copper on lactate metabolism shift in CHO cells.[7] In that study, it was found that higher copper levels in the media shifted lactate producing cultures to lactate consuming cultures due to higher respiratory capacity of the CHO cells, and as a result, antibody titer increased. This finding may explain why copper supplemented cultures in this study exhibited lower lactate levels during late-stage culture (Figure 3e) and slightly higher titer (Figure 4), compared to the copper-free control condition.

Reduced galactosylation observed upon zinc supplement was expected based on previous literature[30] and likely due to reduced intracellular galactosyltransferase activity that occurs as Zn2+/Mn2+ ratio increases.[30] In this case, reduced galactosyltransferase activity due to high zinc concentration may be restored through combined supplement of zinc with Mn2+ or UDP-Gal. Delayed supplement of 100 μM zinc sulphate at 120 h did seem to impact the levels of galactosylated species less by day 7, as cells are likely still adapting to the change in intracellular metal ratio, and the Golgi Apparatus where most of the IgG galactosylation occurs has not yet been fully impacted. Similar glycan peaks across copper-only supplemented cultures demonstrate that galactosylation of the IgG product does not depend on the time of copper supplement. The change in galactosylation due to copper may be a result of differential expression of the B4GALT family genes that encode for β(1,4)-galactosyltransferases, one of the enzymes that catalyzes galactosylation.[31] In a previous study on high versus low copper supplementation in a different CHO cell line, B4GALT3 gene showed differential gene expression.[32] Therefore, this could be one pathway through which copper is taking to reduce galactosylation of the IgG product. The effect of timewise supplementation of copper on charge variants would also be interesting compare here, as previous studies show that higher initial copper concentrations can increase C-terminal proline amidation reaction via peptidyl glycine α-hydroxylating monooxygenase (PAM), which requires copper as a substrate for activity.[8,33] PAM is the only known cuproenzyme to modulate IgG product quality,[33] and to date, no studies have investigated whether delayed addition of copper would correct the shift in basic charge variants in IgG products.

5 | CONCLUSION

This work aimed to investigate how two key trace metals can impact CHO cell growth, metabolism, and antibody production using a response surface methodology (RSM) experimental design approach. Screening time-wise supplementation of these metals revealed that the examined cell line had a higher sensitivity to zinc supplementation regarding the cells' accumulated IgG titer and peak VCD. Addition of combined metals at 0 h seems to ramps up protein production more during the stationary phase compared to when metals are supplemented during or after the exponential growth phase. This work also shows how combined zinc and copper supplementation can further reduce ROS activity (oxidative stress) compared to single metal addition in CHO cells, with copper being the main lever. Glycan analysis revealed the impact of metal addition on glycosylation of the harvested IgG, with further reduction in galactosylation in early combined metal supplemented cultures most likely due to interactions with galactosyltransferases present in the Golgi. Additional investigation to assess the impact of combined metal supplement on other product quality attributes, such as basic or acidic charge variants or sialyation would be advantageous.

The PLS model developed from the DOE run identified some significant factors correlating to cell growth, antibody production, oxidative stress, and specific enzyme activities. Among the three factors contributing to the model, zinc concentration correlated the strongest with most of the response variables, while high copper concentration correlated most with reduced late-stage ROS activity. Response surface optimizer analysis and a validation run confirmed the enhanced IgG specific productivity with maintained cell growth upon combined metal supplementation at ~1:2 ratio of zinc to copper. These findings demonstrate that fine tuning of the concentration and time of zinc and copper (and potentially more trace metals) supplement could be leveraged to further improve the bioprocess performance of CHO cells.

AUTHOR CONTRIBUTIONS

Ashli Polanco: Conceptualization, investigation, manuscript writing – original and finalized draft. **George Liang**: investigation – glycan analysis assay. **Yongdan Wang** and **SoYoung Park**: investigation – cell culture sampling, **Ryan J. Graham**: conceptualization, review and editing of manuscript. **Seongkyu Yoon**: supervision, review and editing manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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PEER REVIEW

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DATA AVAILABILITY STATEMENT

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

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