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RESEARCH ARTICLE

A statistical approach to improve compound screening in cell culture media

Eduard Puente-Massaguer[1] | Llorenç Badiella[2] | Sonia Gutiérrez-Granados[1] | Laura Cervera[1] | Francesc Gòdia[1]

[1]Departament d'Enginyeria Química, Biològica i Ambiental, Campus Bellaterra, Cerdanyola del Vallès, Universitat Autònoma de Barcelona, Barcelona, Spain

[2]Servei d'Estadística Aplicada, Campus Bellaterra, Cerdanyola del Vallès, Universitat Autònoma de Barcelona, Barcelona, Spain

Correspondence
Dr. Eduard Puente-Massaguer, QC-3115, Departament d'Enginyeria Química, Biològica i Ambiental, Edifici Q, Universitat Autònoma de Barcelona, Campus Bellaterra, Cerdanyola del Vallès 08193, Barcelona, Spain.
Email: eduard.puente@uab.cat

The Chinese hamster ovary (CHO) cell line is widely used for the production of recombinant proteins due to its high growing capacity and productivity, as well as other cell lines derived later than CHO. Adapting cell culture media for each specific cell line is a key to exploit these features for cost effective and fast product generation. Media supplementation is generally addressed by means of one-factor-at-a-time or classical design of experiments approaches but these techniques may not be efficient enough in preliminary screening phases. In this study, a novel strategy consisting in folding over the Plackett–Burman design was used to increase cell growth and trastuzumab production of different CHO cell lines through supplementation with nonanimal recombinant compounds. Synergies between compounds could be detected with a reduced number of experiments by using this methodology in comparison to more conventional fractional factorial designs. In the particular case reported here, the sequential use of this modified Plackett–Burman in combination with a Box-Behnken design led to a 1.5-fold increase in cell growth (10×10^6 cells/mL) and a two-fold in trastuzumab titer (122 mg/L) in suspension batch culture.

KEYWORDS
best-fit Box-Behnken, CHO cell line, DoE, folded-over Plackett-Burman, medium optimization, trastuzumab

1 | INTRODUCTION

Today, the most extended practice in the culture of mammalian cells is the use of commercially available chemically defined (CD) and animal-derived component-free culture media. These media have been optimized to support cell growth and recombinant protein production and are usually very complex. However, their composition is not known since they are proprietary. When used for a specific cell line, they may not support all the specific requirements for it, and therefore, additional supplementation of these media with other compounds at optimal concentrations may provide substantial improvement in cell performance.

The CHO cell line is the preferred mammalian cell host for the production of recombinant proteins [1]. Among the multiple benefits on using this system, it should be highlighted that CHO cells can grow in chemically defined and serum-free media enabling standardization of the production process and Good Manufacturing Practices compliance [2]. Also important, CHO cells are easily adapted to grow in suspension culture, which is preferred for large-scale culture [3]. The glycosylation pattern of recombinant proteins produced in engineered CHO cells is very similar to that of human cells. Consequently, protein products can be used in humans since they are nonimmunogenic and bioactive [4]. CHO cells change their chromosome composition at random and frequently, which contribute to their easy adaptation to different culture

Abbreviations: BBD, Box-Behnken Design; CHO, chinese hamster ovary; DoE, design of experiments; ITS, insulin–transferrin–selenium; LOF, lack-of-fit; PBD, Plackett–Burman design; r-, recombinant

conditions and the possibility to find high producer clones through screening [5]. Nowadays, recombinant protein titers from CHO cell culture have improved significantly, reaching the 5-10 gram per liter range [6,7]. In this regard, media supplementation plays an important role in searching for best production conditions since product titer is proportionally related to the number of viable cells in almost all cases [6,8].

In this context, different nonanimal-derived recombinant additives were selected according to their capacities to improve cell culture conditions: r-insulin, r-transferrin, tween 80, selenium, r-albumin, fatty acids, synthetic cholesterol, and (+)-α-tocopherol [9]. Among the most important features of these compounds, insulin acts as a mitogen and growth and maintenance factor in many cell types [10]. Transferrin is a glycoprotein extensively used as an iron chelator in serum-free media and its depletion causes severe inhibition of the cell growth [11]. Tween 80 is a nonionic surfactant and emulsifier frequently used to prevent cell clumping [12]. Interestingly, it has also been recently reported that it improves protein production in different mammalian cell types [13]. Selenium is an essential trace element for normal cell growth and development. It is incorporated into enzymes that protect cells by reducing peroxides fundamentally acting as an antioxidant [14]. Human serum albumin can also acts as an antioxidant, as well as playing an important role in binding and transporting physiologically important ligands [15]. Fatty acids and synthetic cholesterol are normally required for cell growth since they are the main constituents of the phospholipidic cell membrane [16]. Finally, (+)-α-Tocopherol acetate, also known as vitamin E, is an important lipid that helps to stabilize the cellular membrane and span the viability of the cell culture [17].

The assessment of such a broad number of compounds may results in a tedious procedure, if not evaluated with efficient methodologies. Classical one-factor-at-a-time approaches can be costly and time consuming and multiple factorial effects cannot be identified and are not considered. To accelerate this phase, the use of DoE-based statistical methodologies is an adopted strategy in medium development and optimization [18]. In a preliminary phase with many variables to be analyzed, an efficient way to screen for the important factors with a limited number of experiments is the use of 2^k factorial designs. However, full factorial and 2^k fractional designs require a high experimental effort when there are many k factors to be analyzed [19]. In this case, D-optimal Plackett–Burman designs (PBDs) become a useful alternative [20]. Still, PBDs are saturated designs developed to eliminate factors potentially having no or negligible effects over the responses. This means that only main effects are to be considered, which implies to ignore aliasing patterns due to higher order interactions [20]. Rejecting this possible scenario, which is very common in media optimization [21,22], may guide upcoming experimentation to an incorrect decision. In this study, a folded-over matrix of the conventional PBD was implemented to improve the detection of factor interactions in the screening phase of a full DoE approach. The results obtained by the application of this technique were compared to the classical methodology.

After the identification of significant compounds influencing the response variable, the next step in a full DoE process is the optimization of their concentrations through response surface methodologies [23,24]. Most widely used response surface methodology include Central Composite Designs (CCDs) and Box-Behnken Designs (BBD). The selection among any of these methodologies is based on the number of k factors to be evaluated, the design space, and the optimality criteria [25]. For $k = 3$, Central Composite Designs require a higher number of experimental runs than the rest of designs [19] and it has also been demonstrated that BBDs are slightly more efficient [26]. Accordingly, a BBD was selected for optimization and the obtained polynomial equation was subjected to a best-fit iteration approach toward defining the smallest combination of significant compounds contributing to maximizing the response.

The work presented here combines the sequential use of a folded-over PBD toward detecting possible synergies between different compounds in the screening phase followed by a best-fit BBD to optimize their concentrations. The optimum supplementation cocktail was validated and also tested in another CHO cell line stably expressing trastuzumab, an antitumoral monoclonal antibody against human breast

PRACTICAL APPLICATION

In animal cell-based processes in the biopharmaceutical industry, media supplementation tailored to each cell line is nowadays a very relevant component for production optimization. A widely approach followed toward media optimization is to first use a screening methodology, normally a Plackett–Burman design (PBD), to discard compounds with no or negligible effects on cell culture. However, the classical PBD cannot distinguish between the effects due to individual factors and interactions, which may be critical in next experimentation phases. In this work, the authors propose a folding-over strategy of the PBD in order to be able to detect these synergies between factors. Also, a comparison between the classical PBD and the technique here proposed is shown to have significant differences. By using this methodology, the authors envisage a window of applicability for media supplementation since more robust decisions can be undertaken during the phase of compound screening.

cancer [27], in the same culture conditions in order to value the applicability of this compound combination.

2 | MATERIALS AND METHODS

2.1 | Cell line, media, and culture conditions

The cell line used in the screening and optimization steps in the present work is a serum-free suspension-adapted CHO-S cell line (ThermoFisher Scientific, Waltham, MA, USA). Also, a serum-free suspension-adapted CHO-S cell line expressing trastuzumab (Cobra Biologics AB, Keele, UK) was tested with the optimal condition obtained in this study. Two commercial serum-free media formulations for CHO-S were tested for cell growth. These include ProCHO5 (Lonza Biologics, Basel, Switzerland) and FreeStyle-CHO (ThermoFisher Scientific, Waltham, MA, USA). All formulations were supplemented with GlutaMAX (8 mM) (ThermoFisher Scientific, Waltham, MA, USA). Cell cultures were preadapted to each formulation prior to experimentation. Cells were routinely maintained and passed every 2–3 days in 125 mL disposable polycarbonate Erlenmeyer flasks (Corning, Steuben, NY, USA) in 20 mL of culture medium. Flasks were shaken at 130 rpm using an orbital shaker (Stuart, Stone, UK) placed in an incubator maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

Cell count and viability were determined using a Nucleocounter NC-3000 (Chemometec, Allerod, Denmark) during 8 days. The maximum specific growth rate, μ_{max} (h^{-1}), and duplication time, $t_{1/2}$ (h) were determined from the data corresponding to the exponential growth phase.

A YSI 2700 Select glucose/lactate analyzer (YSI, Yellow Springs, OH, USA) was used to measure the concentrations of glucose and lactate in cell culture supernatants.

2.2 | Toxicity assays of the compounds

Cell Titer 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was used to perform toxicity assays of all the compounds studied according to manufacturer instructions. Briefly, 100 μL of CHO-S cells was inoculated at 0.3 million cells/mL in a 96-well plate (Nalgene Nunc International, Rochester, NY, USA) together with 10 μL of the studied compound at different concentrations. After 48 h, 20 μL of Cell Titer 96 reagent was added to each well and then the 96-well plate was incubated for 1 h at 37°C and at 130 rpm orbital shaking. A calibration curve of known cell concentration (from 0 to 1.5×10^6 cells/mL) was performed in duplicate just before the analysis. The absorbance was measured in a Victor^3 spectrophotometer (PerkinElmer, Waltham, MA, USA) at a wavelength of 490 nm.

2.3 | Compound preparation

Nonanimal derived medium compounds used for media supplementation included three recombinant proteins: r-albumin (Merck Millipore, Kankakee, IL, USA), r-transferrin (Merck Millipore, Billerica, MA, USA), and r-insulin (Novo Nordisk Pharmatech A/S, Køge, Denmark); sodium selenite (selenium), synthetic cholesterol, fatty acids, (+)-α-tocopherol acetate, and tween 80 (Sigma, St. Louis, MO, USA). r-Transferrin solution was prepared from 20 mg/mL commercial stock in sterile PBS. r-Insulin, r-albumin, and selenium solutions were prepared from commercial powder stocks in PBS and subsequently 0.22 μm sterile filtered. Synthetic cholesterol and fatty acids solutions were prepared in PBS from 500*X* and 2000*X* commercial stocks, respectively. Tween 80 and (+)-α-tocopherol acetate were prepared from pure commercial stocks in sterile PBS to a concentration of 25 mg/mL and 2 mg/mL, respectively, both of them referred as 1000*X*.

2.4 | Medium optimization using DoE

The screening of the eight nonanimal-derived medium compounds was performed with a folded-over PBD while BBD was chosen to optimize the concentration of the compounds with positive effect on cell growth. CHO-S cells were seeded at a cell density of 0.3×10^6 cells/mL in all DoE experiments and growth kinetics was followed every 24 h during 8 days, thus, allowing to determine the maximum viable cell concentration reached for each experimental condition tested.

2.5 | Plackett–Burman design

A two-time replicated 24-run folded-over PBD (Table 1) was used to identify the compounds with a significant effect on CHO-S cell growth and screen out negligible factors [20]. The eight compounds were screened at two levels: a low level (no additive) coded as −1 and a high level coded as +1. High levels for each factor were defined based on the toxicity experiments (Fig. 1) and on pre-existing knowledge from the literature [28]. To this purpose, 24-h amplified cells were split into 48 shake flasks with fresh medium and then the different compound combinations were added. Compound concentrations used for the PBD are provided in Supporting Information 1, whereas the experimental design matrix in coded values and maximum viable cell concentration (10^6 cells/mL) are shown in Table 1.

The effect of each experimental variable upon the measured response (viable cell concentration) was determined as the difference between the average responses at the high level (+1) and the average responses at the low level (−1), as shown in Equation 1:

$$E_{ij} = \frac{\sum Rj\,(+1)}{n/2} - \frac{\sum Rj\,(-1)}{n/2} \quad (1)$$

TABLE 1 Matrix design in coded levels, response and ANOVA analysis for the Plackett–Burman design with all factors and the best-fit model

Exp no.	Selenium	r-Transferrin	r-albumin	r-Insulin	Tocopherol	Tween 80	Fatty acids	Synthetic cholesterol	Response a	Response b
1	−1	+1	−1	+1	−1	−1	−1	−1	8.58	7.96
2	+1	+1	+1	−1	+1	−1	+1	+1	9.31	9.07
3	+1	−1	+1	−1	−1	−1	−1	+1	6.12	6.42
4	−1	−1	−1	+1	+1	+1	+1	+1	6.72	6.44
5	−1	−1	−1	−1	+1	+1	+1	+1	6.23	5.72
6	+1	+1	−1	+1	−1	+1	+1	−1	9.6	8.99
7	−1	−1	−1	−1	−1	−1	−1	−1	7.15	6.84
8	−1	+1	−1	−1	−1	−1	+1	+1	10.30	10.10
9	−1	+1	+1	−1	−1	+1	+1	−1	10.30	9.57
10	+1	−1	+1	−1	+1	+1	−1	−1	6.50	6.86
11	+1	−1	−1	−1	−1	+1	+1	+1	6.05	5.39
12	−1	+1	+1	+1	+1	+1	−1	+1	8.12	7.60
13	+1	+1	+1	+1	+1	−1	+1	−1	8.32	9.11
14	+1	+1	−1	−1	+1	−1	+1	−1	9.48	7.80
15	−1	−1	+1	+1	+1	+1	+1	−1	5.96	6.55
16	−1	−1	+1	+1	−1	−1	+1	−1	6.21	6.97
17	+1	−1	+1	+1	−1	−1	+1	+1	6.71	5.87
18	+1	−1	−1	+1	+1	−1	−1	+1	6.30	7.46
19	−1	−1	+1	−1	+1	−1	−1	−1	7.14	-
20	+1	+1	−1	−1	+1	+1	−1	−1	8.88	7.86
21	+1	+1	+1	+1	−1	+1	−1	+1	9.15	8.59
22	+1	−1	−1	+1	−1	+1	−1	−1	7.86	8.36
23	−1	+1	−1	+1	+1	−1	−1	+1	9.97	8.92
24	−1	+1	+1	−1	−1	+1	−1	+1	8.96	7.40

Factors	Coefficient	*t*-Value	*p*-Value
Constant	6.28	1.26	<0.01
r-Transferrin	0.77	0.89	0.38
r-Insulin	1.43	2.28	0.03
Tween 80	−2.75	−4.38	<0.01
r-Transferrin · r-Insulin	−0.75	−1.90	0.06
r-Transferrin · Tween 80	1.78	4.49	<0.01

Responses a and b are maximum viable cell concentration values from duplicate experiments in millions of cells/mL.

where E_{ij} is the effect of the variable i on a response j, Rj is the measured response j, and n is the number of experimental runs. A positive value for E_{ij} means that the variable i increases response j if added at the high level, and vice versa.

Plackett–Burman experimental results were fitted to a first-order polynomial function with and without considering interactions by linear regression analysis as described below:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i \cdot X_i + \sum_{i=1}^{k} \sum_{j>1}^{k} \beta_{ij} \cdot X_i \cdot X_j + \varepsilon \qquad (2)$$

where Y is the response variable (cell density in million cells/mL), k is the number of factors, β_0 is the model intercept term, β_i the linear coefficient of the different parameters which correspond to one-half of the corresponding factor estimates, β_{ij} stands for the interaction coefficient, X_i and X_j are the studied compounds, and ε is the error associated to experimentation.

2.6 | Box-Behnken design

In order to define the optimal concentration for each factor selected in the screening phase, a Box-Behnken design was used. Compounds were screened at three equidistant levels: a low level coded as −1, a medium level coded as 0, and a high level coded as +1, as indicated in Supporting Information 2. To do this, 24-h amplified cells were split into 30 shake flasks with fresh medium and the different

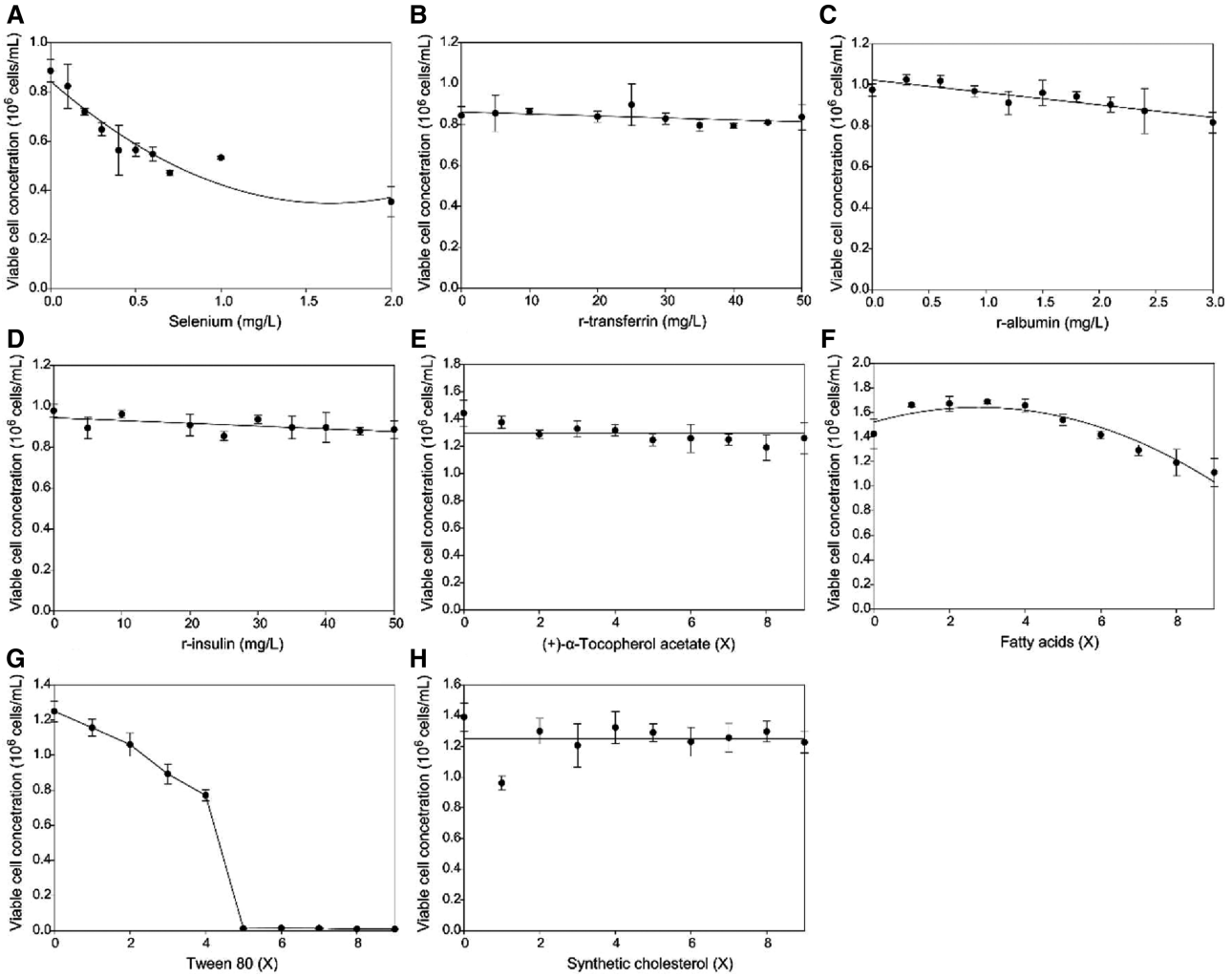

FIGURE 1 Toxicity assay curves for the different supplements in CHO-S cells; (A) selenium (mg/L); (B) r-transferrin (mg/L); (C) r-albumin (mg/L); (D) r-insulin (mg/L); (E) (+)-α-Tocopherol acetate (*X*); (F) fatty acids (*X*); (G) Tween 80 (*X*); (H) synthetic cholesterol (*X*)

compound combinations were added. Table 2 outlines the experimental design matrix in coded values, the response, and the statistical analysis of the method. The obtained results were fitted to a second-order polynomial equation by a linear regression analysis and subjected to an iteration-based refinement process (Equation (3)):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i \cdot X_i + \sum_{i=1}^{k} \beta_{ii} \cdot X_i^2 + \sum_{i=1}^{k} \sum_{j>1}^{k} \beta_{ij} \cdot X_i \cdot X_j + \varepsilon \quad (3)$$

where Y is the response variable (cell density in million cells/mL), β_0 is the model intercept term, β_i the linear coefficient, β_{ii} the quadratic coefficient, β_{ij} the interaction coefficient, X_i and X_j are the studied compounds, and ε is the error associated to experimentation. The model Equation (3) was used to predict the optimal concentrations of the selected compounds using the L-BFGS-B quasi-Newton algorithm implemented in the *optimx* package of the R software (R Development Core Team, Vienna, Austria). Three-dimensional plots were generated according to the equations toward facilitating model interpretation.

2.7 | Statistical analysis

Statistical analyses of the models for PBD and BBD were performed using R Software with the *Frf2* and *car* packages. This license-free software was also used to develop the corresponding figures of the study. The quality of the regression of the model equations was evaluated by the coefficients R^2 and R^2_{adj}. The validity of the equations was also checked by comparing the differences associated to experimental and pure error through the lack-of-fit (LOF) test ($\alpha = 0.05$). The overall significance was determined with the ANOVA *F*-test ($\alpha = 0.05$), whereas the significance of each coefficient was determined by the corresponding *t*-test ($\alpha = 0.05$). Finally,

TABLE 2 Matrix design, response and ANOVA analysis for Box-Behnken experimental design of the different supplements: r-transferrin (X_1), r-insulin (X_2), tween 80 (X_3)

Exp no.	r-Transferrin	r-Insulin	Tween 80	Response a	Response b
1	−1	−1	0	8.17	7.82
2	1	−1	0	10.5	10
3	−1	1	0	8.71	7.84
4	1	1	0	7.8	7.95
5	−1	0	−1	5.8	6.14
6	1	0	−1	7.62	7.50
7	−1	0	1	5.48	5.56
8	1	0	1	5.06	4.36
9	0	−1	−1	7.4	8.8
10	0	1	−1	7.62	8.01
11	0	−1	1	8.19	6.68
12	0	1	1	7.83	7.12
13	0	0	0	8.09	8.12
13	0	0	0	7.74	7.67
13	0	0	0	7.53	7.83

	Coefficient	*t*-Value	*p*-Value
Constant	7.83	35.53	<0.01
r-Transferrin	0.32	2.43	0.02
r-Insulin	−0.29	−2.17	0.04
Tween 80	−0.54	−3.98	<0.01
r-Transferrin·r-Insulin	−0.66	−2.52	<0.01
r-Transferrin·Tween 80	−0.48	6.39	<0.01
r-Transferrin^2	−0.50	−7.01	<0.01
r-Insulin^2	1.27	−3.48	<0.01
Tween 80^2	−1.39	−3.13	<0.01

All the variables were studied at three levels: a low level coded as −1, a medium level coded as 0, and a high level coded as +1.
Responses a and b are maximum viable cell concentration values from duplicate experiments (runs 1–12) in millions of cells/mL. Run 13 was performed in triplicate because it was the centre point.

normality of the calculated residuals was evaluated by means of the Shapiro–Wilk test ($\alpha = 0.05$).

2.8 | Trastuzumab quantification

Trastuzumab concentration was determined by the commercial solid-phase enzyme-linked immunosorbent assay SHIKARI Q-TRAS (Matriks Biotechnology, Ankara, Turkey) according to manufacturer instructions. Briefly, 100 μL of Assay Buffer was added into each of the 96-well plate wells to be used. A total of 10 μL of each diluted sample and ready-to-use trastuzumab standards were added to the plate in triplicate. After 30 min of incubation at room temperature, the incubation solution was discarded and the plate was washed three times. A total of 100 μL of peroxidase conjugate was added into each well. After 60 min of incubation at room temperature, the incubation solution was discarded and the plate was washed three times. Also, 100 μL of TMB substrate solution was added into each well and the plate was incubated in the dark for 10 min. The colorimetric substrate reaction was stopped by adding 100 μL of Stop solution and absorbance was measured at 450 nm in a Victor^3 spectrophotometer (Perkin Elmer).

3 | RESULTS

3.1 | Selection of the medium for supplementation

ProCHO5 and FreeStyleCHO serum-free commercial media specific for CHO suspension culture were selected considering their use in industrial processes [29,30]. Cell growth of CHO-S cells was firstly characterized in these two media before starting with the sequential DoE approach. In both cases, cells maintained a high viability (> 90%) with a doubling time between 24 and 31 h at the exponential phase (Fig. 2A). A maximum viable cell concentration of 5.2 ± 1.0 × 10^6 cells/mL was attained between 96 and 120 h in

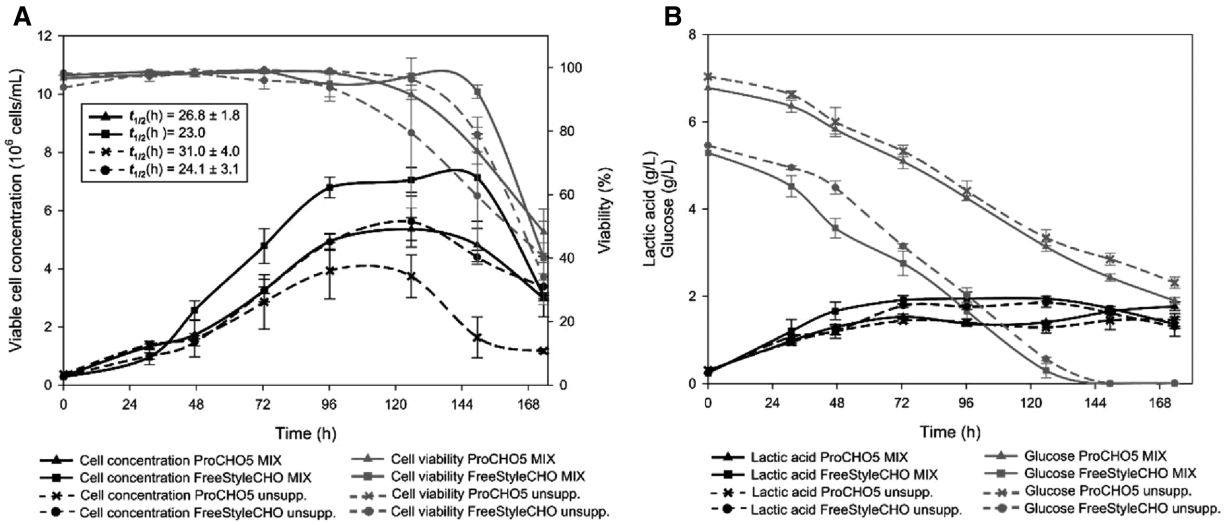

FIGURE 2 (A) Growth kinetics of CHO-S cells in batch culture in different culture media with and without MIX supplementation. Cells were seeded at 0.3×10^6 cells/mL in 125-mL flasks in their growing exponential phase. Cell density and viability of each culture were determined at every 24 h. (B) Lactic acid and glucose profiles in the different culture media with and without MIX supplementation. Mean values ± standard deviation of triplicate experiments are represented

FreeStyleCHO medium, whereas ProCHO5 reached 3.6 ± 0.9×10^6 cells/mL. When cell viability started to decrease, the availability of glucose was not limiting in ProCHO5 medium (> 1.5 g/L) while it was in FreeStyleCHO (Fig. 2B). However, adding a bolus feed of glucose to avoid this limitation did not improve cell growth (data not shown), hence, indicating limitation by other factors in this medium. In respect to lactate production, it did not exceed 2 g/L in any media tested, which is typically considered the toxic concentration [31].

The two commercial media were then supplemented with a mixture of various compounds, here referred as MIX, in a concentration previously optimized for the HEK293SF-3F6 cell line [32]. This mixture included r-insulin (19.8 mg/L), r-transferrin (1.6 mg/L), (+)-α-tocopherol acetate (0.9*X*), tween 80 (0.9*X*), synthetic cholesterol (0.9*X*), and fatty acids (0.9*X*). As the concentration of the different compounds was not optimized for CHO cells, this experiment only consisted in a proof of concept to elucidate if the addition of these compounds to the media could improve maximum cell concentration. In all cases, cells maintained a high viability (> 90%) and the doubling time was reduced compared to nonsupplemented conditions (Fig. 2A). Also, an increase in maximum cell concentration was evident upon supplementation, 1.3-fold in FreeStyleCHO (6.7 ± 0.7 × 10^6 cells/mL) and 1.5-fold in ProCHO5 (5.4 ± 0.4 × 10^6 cells/mL). In these conditions, lactate production did not exceed 2 g/L and it was consumed only in FreeStyleCHO medium since 120 h.

According to CHO cell behavior in each formulation, FreeStyleCHO was the medium selected for optimization since cells reached a higher concentration with a reduced doubling time.

3.2 | Toxicity assays for the compounds tested as supplements

Toxicity studies for each one of the individual compounds were performed in order to define the concentration ranges for the PBD.

Maximum viable cell concentrations after supplementation with varying concentrations of each compound are presented in Fig. 1. Fatty acids, tween 80, selenium, and to a lesser extent r-albumin were the compounds showing some cell growth inhibition in the concentration ranges tested. These tests were used in combination with literature to properly set a suitable design space to perform the PBD (Supporting Information 1).

3.3 | Compound screening using the folded-over PBD

A folded-over PBD of experiments was used as the screening methodology to find the significant compounds affecting cell growth. Two independent replications ($n = 2$) were performed for each experimental run in the matrix so as to calculate pure error.

A first analysis of the PBD_{12+12} results showed that linear regression without considering two-factor interactions could not accurately fit the experimental observations. The poor LOF test associated *p*-value of 0.004 indicated that only individual effects could not effectively explain data distribution. This suggested that effects firstly attributed to individual factors could be aliased with interactions between factors and that data from the PBD had to be analyzed considering them. Linear regression combining main and interaction effects showed

that the model was more efficient to fit the experimental data:

$$\begin{aligned}\text{Cell concentration}\,(10^6\text{cells/mL}) = {} & 6.28 + 0.77 \cdot [\text{r} - \text{Transferrin}] \\ & +1.42 \cdot [\text{r} - \text{Insulin}] \\ & -2.75 \cdot [\text{Tween80}] \\ & -0.75 \cdot [\text{r} - \text{Transferrin}] \cdot [\text{r} - \text{Insulin}] \\ & +1.78 \cdot [\text{r} - \text{Transferrin}] \cdot [\text{Tween80}]\end{aligned} \quad (4)$$

A nonsignificant LOF test p-value = 0.43, a R^2 coefficient of 0.81 and a R^2_{adj} of 0.79 corroborated that the model containing individual effects and interactions fitted well. The statistical significance of the model was confirmed with an F-test associated p-value < 0.01 and each regression coefficient was analyzed with the Student's t test (Table 1). The Shapiro–Wilk test of the residuals confirmed the normality assumption of the data since the null hypothesis was not rejected (p-value = 0.85). The predictive capability of the model was also assessed with the predicted error sum of squares value, which led to the calculation of a *pred*. R^2 coefficient of 0.75, very close to the R^2_{adj}.

The comparison between the classical PBD_{12}, only based on individual effects, and the new approach here presented is shown in Fig. 3A-B, respectively. Main effect plots of the different compounds in both strategies include a color coding based on the Student's t test associated p-value. Student's t test associated p-values lower than 0.05 (dark grey) imply that the compound is relevant whereas p-values > 0.05 (light grey) mean that there are no evidences to declare the factor as important. The classical approach showed r-transferrin as the only factor affecting cell growth, whereas the model containing interactions showed r-insulin and two two-way interactions comprising r-transferrin/r-insulin and r-transferrin/tween 80 as significant. The sole addition of tween 80 and the small interaction between r-transferrin and r-insulin proved to be deleterious for cell growth. However, the higher positive effects of adding r-insulin and the combination of r-transferrin and tween 80 counteracted the weak r-transferrin/r-insulin interaction and the negative effect of tween 80 by itself, respectively. Taking into account all these considerations, r-transferrin, r-insulin, and tween 80 were chosen for subsequent optimization.

3.4 | Optimization of compound concentrations using the Box-Behnken design

An independently replicated (n = 2) three-factor Box-Behnken design was used to further optimize the concentrations of r-transferrin, r-insulin, and tween 80 in the culture medium. In this phase, the range of r-insulin concentrations was reduced since no improvement in cell growth was observed beyond 2 mg/L.

Maximum viable cell concentrations for each experimental run were fitted to a second-order polynomial as described in Equation 3. The obtained equation was subjected to a refinement process to find the best fitting with the lowest number of parameters:

$$\begin{aligned}\text{Cell concentration} = {} & 7.90 + 0.27 \cdot [\text{r} - \text{Transferrin}] \\ & -0.29 \cdot [\text{r} - \text{Insulin}] \\ & -0.47 \cdot [\text{Tween80}] \\ & -0.66 \cdot [\text{r} - \text{Transferrin}] \cdot [\text{r} - \text{Insulin}] \\ & -0.48 \cdot [\text{r} - \text{Transferrin}] \cdot [\text{Tween80}] \\ & -0.59 \cdot [\text{r} - \text{Transferrin}]^2 \\ & +1.30 \cdot [\text{r} - \text{Insulin}]^2 \\ & -1.49 \cdot [\text{Tween80}]^2\end{aligned} \quad (5)$$

Regression analysis showed that the model was adequate with a coefficient R^2 of 0.88, which suggested that 88% of the variability in the data was explained by the model Equation (5) (Fig. 3C). The R^2_{adj} value of 0.83 and the *pred*. R^2 of 0.75 further corroborated model adequacy. The statistical significance of the model was confirmed with an F-test associated p-value < 0.01. The significance of each regression coefficient of the best-fit model was analyzed by Student's t test and is shown in Table 2. In all cases, Student's t test associated p-values < 0.05 and, thus, all terms were considered significant. The study of the residuals by means of the Shapiro–Wilk test confirmed the normality assumption of the data (p-value = 0.23). In conclusion, the ANOVA analysis of the model Equation 5 was satisfactory for the experimental data obtained with the BBD and the model could be used to navigate the design space.

3.5 | Response surface analysis of the BBD

Three-dimensional plots were constructed for visual analysis of the response trend regarding different combinations of r-insulin, r-transferrin, and tween 80 (Fig. 4).

The multifactorial nature of this optimization could be observed in the two optima identified by solving the second-order polynomial Equation (5) (Supporting Information 3). These two different compound combinations provided the highest levels of cell concentration, one with a high level of r-transferrin and another with the highest level of r-insulin and a moderate level of r-transferrin (Fig. 4A-C). Of note, the negative interaction between r-transferrin and r-insulin previously encountered in the PBD was also present in the BBD (Fig. 4E). As r-transferrin concentration increased, the optimum condition moved from higher to lower r-insulin concentrations (Fig. 4D-F). Tween 80 levels were similar in both optima and around the middle of the evaluated concentration range (Fig. 4G-I).

The optimum with r-transferrin reaching very high levels (57 mg/L) was considered difficult to be adopted in practical terms due to the high cost of r-transferrin. Therefore, the optimum condition making a higher significance was the one

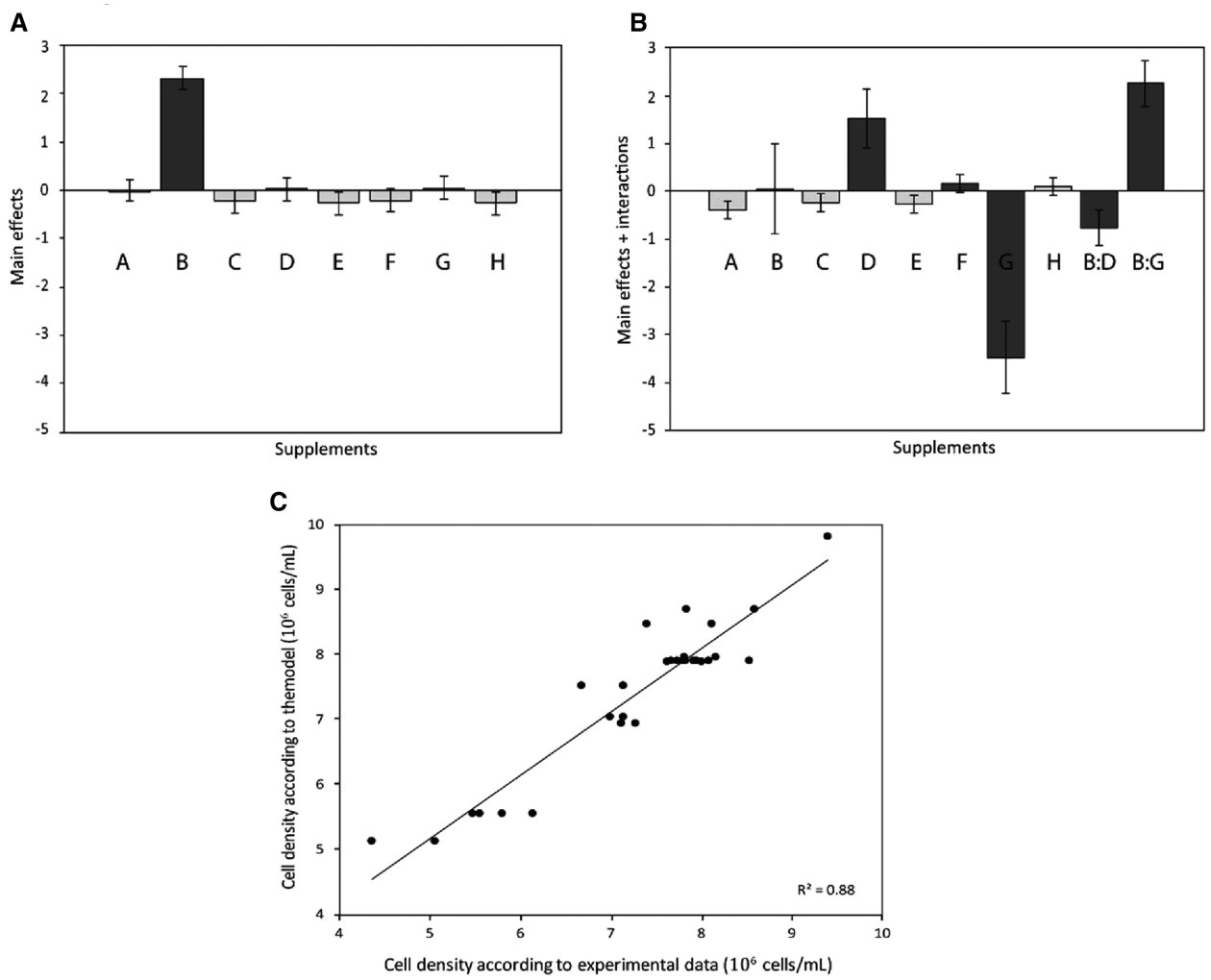

FIGURE 3 (A) Main effects plot of the PBD only considering main effects of non-animal derived supplements on CHO-S cells in FreeStyleCHO medium, where: A, selenium (mg/L); B, r-transferrin (mg/L); C, r-albumin (mg/L); D, r-insulin (mg/L); E, (+)-α-Tocopherol acetate (X); F, fatty acids (X); G, Tween 80 (X); H, synthetic cholesterol (X). Dark grey bars represent significant factors (p-value < 0.05) whereas the light grey ones mean nonrelevant factors (p-value > 0.05). The response variable is maximum viable cell concentration (10^6 cells/mL). (B) Main effects plot of the PBD considering main effects with relevant interactions of the different compounds. (C) Correlation between the experimental data and Box-Behnken model predicted cell densities. Mean values ± standard deviation of duplicate experiments are represented

with 21.3 mg/L of r-transferrin, 2 mg/L of r-insulin, and 1.8X of tween 80. Under these conditions, the predicted maximum viable cell concentration was $9 \pm 1.1 \times 10^6$ cells/mL.

3.6 | Validation of the model

A validation experiment was carried out to corroborate the optimal supplementation conditions predicted by the second order polynomial. A maximum viable cell concentration of $10 \pm 0.6 \times 10^6$ cells/mL ($n = 3$) was attained under these conditions (Fig. 5A), close to the model prediction ($9 \pm 1.1 \times 10^6$ cells/mL), confirming model accuracy. Of note, a 1.5-fold improvement was obtained for CHO-S cells growing in the optimum condition compared to nonsupplemented FreeStyleCHO medium ($6.6 \pm 0.4 \times 10^6$ cells/mL). A better adaptation to the supplemented medium could also be observed since cell-doubling time was significantly shortened from 23 to 20 h (Student's t test associated p-value = 0.048).

3.7 | Supplementation of a trastuzumab expressing CHO-S cell line

In order to generalize the results obtained with the application of this new DoE approach, a CHO-S cell line expressing trastuzumab was used in the same culture conditions. In this context, 121.8 ± 6.7 mg/L was obtained, which represented a two-fold increase when compared to nonsupplemented FreeStyleCHO medium (62.5 ± 15.3 mg/L) as observed in Fig. 5B. A maximum cell concentration of $8.8 \pm 0.2 \times 10^6$ cells/mL was obtained upon supplementation, a 1.7-fold

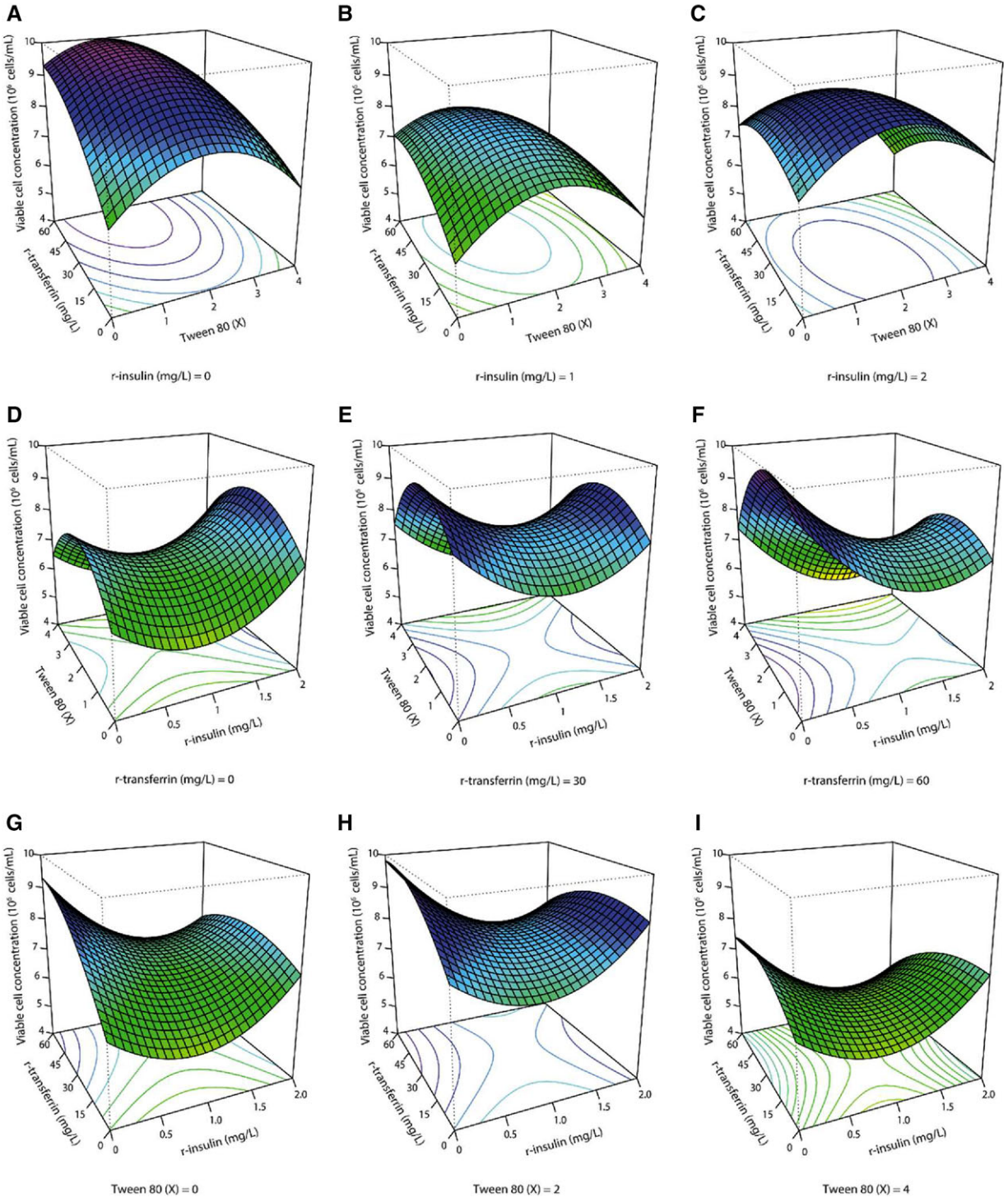

FIGURE 4 Response surface graphs based on Box-Behnken experimental results (A–I). Maximum viable cell concentration in cell culture as a function of the concentrations of (A–C) r-transferrin versus tween 80; (D–F) tween 80 versus r-insulin; and (G–I) r-transferrin versus r-insulin. The graphs were constructed depicting two variables at a time while keeping the third one at a fixed level

increase when compared to the nonsupplemented medium ($5.1 \pm 0.3 \times 10^6$ cells/mL). As for the nonexpressing CHO-S cell line, the doubling time significantly decreased from 24 to 20 h in supplemented conditions (Student's t test associated p-value = 0.008).

4 | DISCUSSION

In this study, a powerful approach for compound screening based on the use of a folding-over PBD was successfully used with the objective to improve cell growth and production in

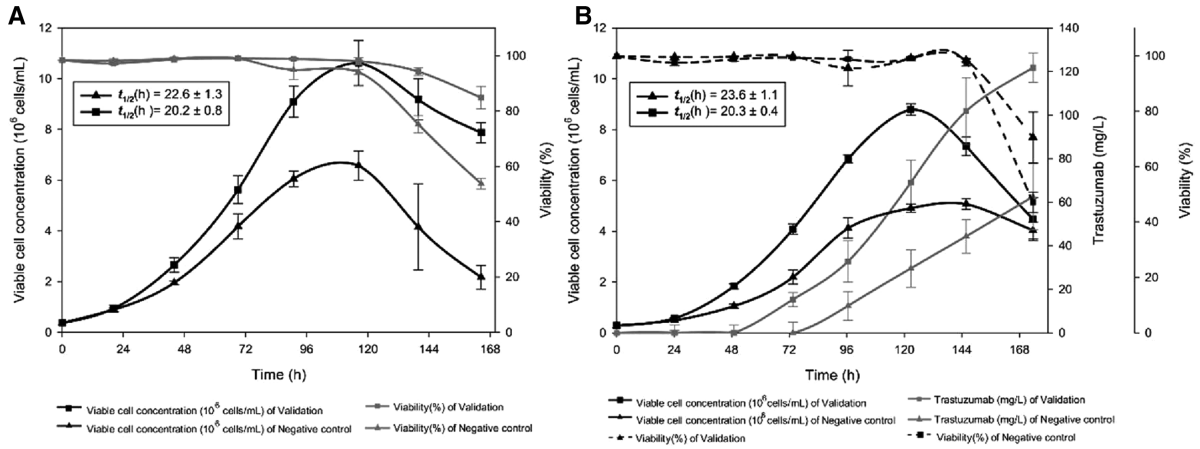

FIGURE 5 (A–B) Model validation. (A) Unsupplemented (Negative control) and supplemented CHO-S cell line in FreeStyleCHO medium with the optimal levels of r-transferrin, r-insulin and tween 80. (B) Non-supplemented and supplemented CHO-S cells expressing transtuzumab in FreeStyleCHO medium with the optimal levels of r-transferrin, r-insulin and tween 80. Cell density and viability were determined daily. Mean values ± standard deviation of triplicate experiments are represented

CHO-S cells. The use of this methodology in a preliminary phase of a full DoE approach enabled the detection of some compounds that were negligible when considered individually but they were not when used in combination.

Considering the increasing pressure on cell culture-based processes in terms of production, media optimization is of interest especially if a systematic approach is followed in its definition [9]. Media supplementation is more affordable for the majority of cell culture users compared to the development of an in-house formulation. The latter normally includes a lot of different compounds and the effort required in time and resources is very high. Commercial media is a good alternative to full media development, however, their composition is not known and they are developed for a scope of cell lines. Therefore, there is an opportunity for supplementation to substantially improve the yields in cell concentration and product titer by relatively modest efforts.

A general approach toward media optimization is to first use a screening design to select the relevant compounds showing a positive effect on cell culture [33]. In this sense, the resolution III PBD is an accepted methodology that fits to this purpose. However, most publications related to media optimization do not explore further in the nature of the effects caused by each of the compounds [34,35]. Aliasing arising from the synergies between the different compounds with the main individual effects may compromise the interpretation of the results, which is often not considered in the early steps of media supplementation [36]. This is a critical decision, which may guide the next steps in experimentation to an incorrect decision (i.e. optimization designs). The classical PBD contains an alias structure between main effects and two-factor interactions that can be resolved by folding-over the design. To do this, the Hadamard's matrix of the PBD is increased with the opposite combinations of the initial runs. Alternative designs, such as the 2^{8-4}_{IV} or the 2^{8-3}_{V} fractional factorials either cannot untangle two-factor interactions or they require more experimental runs compared to the 24-run folded-over PBD, respectively. The key to folding over is choosing all the individual factors considered in the PBD_{12+12} and perform a linear regression. If LOF test associated p-value is significant, there is evidence that interactions are relevant and need to be considered in the model [37]. The determination of these interactions is based on a trial and error process considering the effect heredity, hierarchy, and sparsity principles [38]. The first rule states that it is most probable for an interaction to be active if at least one of the factors involved has an active main effect. The second and third principles assume that lower order is more important than higher order effects and that the number of relatively important factors is small, respectively [39]. The comparison of the folded PBD_{12+12} to the classical PBD_{12} revealed the aliasing pattern between r-transferrin and the combination of r-transferrin with r-insulin and r-transferrin with tween 80, which could not be detected by the classical PBD approach.

Using the folded-over PBD in combination with a BBD defined an optimum supplementation cocktail of $1.8X$ of tween 80, 21.3 and 2 mg/L of r-transferrin and r-insulin, respectively. Under these conditions, a maximum viable cell concentration of 10×10^6 cells/mL was obtained in batch culture, representing a 1.5-fold increase in maximum cell concentration compared to the nonsupplemented medium. The optimum condition was also used in a CHO-S cell line stably expressing trastuzumab with a 1.7-fold improvement of cell growth and 2-fold increase in final antibody titer. A comparison of the specific antibody productivity (q_p) revealed that the improvement in trastuzumab concentration was a consequence of a higher cell concentration and not of an increase in q_p. Indeed, the comparison of both specific antibody

productivities based on trastuzumab titer and the integral of viable cell concentration resulted in $1.3 \pm 0.3 \times 10^{-10}$ for the supplemented medium and $1.2 \pm 0.4 \times 10^{-10}$ mg/(cell · h) for the nonsupplemented one. Student's *t*-test associated *p*-value of 0.91 indicated that there were no significant differences between both conditions in terms of specific antibody production. Of note, efficient supplementation of commercial media for a specific cell line is paramount toward maximizing the final protein titer and reducing the production costs.

Insulin and transferrin in combination with other compounds have been reported to have a variety of positive effects on mammalian cell culture. For example, combining them with selenium (ITS) in CHO-DG44 cells improved cell growth by a 35% [40]. The only combination of insulin and transferrin had also proven to increase 18% TNFR-Fc production in a GS-CHO cell line [41] and prourokinase production in 11G-S CHO cells [42]. Tween 80 has recently been demonstrated to have a beneficial effect on mAb production in CHO-K1 and CHO DUX-B11 [13]. The authors mention that the interest in using tween 80 relies on its capacity to extend the stability of media compounds in solution for a longer period of time, which spans their availability in the culture medium. In this regard, the combination of tween 80 with r-insulin and r-transferrin is shown as an interesting supplementation cocktail for CHO-S-derived cell lines compared to reported insulin and transferrin combinations, in which lower growth and production yields are achieved [40,41].

In conclusion, an advantageous approach for compound screening and medium supplementation was successfully developed in this work. This methodology deepened in the synergies between compounds at early screening stages and highlighted the importance of considering these factor interactions in later experiments.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

Ethical approval

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

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