

A semi-empirical glycosylation model of a camelid monoclonal antibody under hypothermia cell culture conditions

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Abstract The impact of cell culture environment on the glycan distribution of a monoclonal antibody (mAb) has been investigated through a combination of experiments and modeling. A newly developed CHO DUXB cell line was cultivated at two levels of initial Glutamine (Gln) concentrations (0, 4 mM) and incubation temperatures of (33 and 37 °C) in batch operation mode. Hypothermia was applied either through the entire culture duration or only during the post-exponential phase. Beyond reducing cell growth and increasing productivity, hypothermia significantly altered the galactosylation index profiles as

compared to control conditions. A novel semi-empirical dynamic model was proposed for elucidating the connections between the extracellular cell culture conditions to galactosylation index. The developed model is based on a simplified balance of nucleotides sugars and on the correlation between sugars' levels to the galactosylation index (GI). The model predictions were found to be in a good agreement with the experimental data. The proposed empirical model is expected to be useful for controlling the glycoprofiles by manipulating culture conditions.

Keywords Chinese hamster ovary cells · Monoclonal antibody · Hypothermia · Glycosylation · Mathematical modeling

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Abbreviations

[Ala]	Alanine concentration (mM)
[AM]	Ammonia concentration (mM)
[Asn]	Asparagine concentration (mM)
[Asp]	Aspartic acid concentration (mM)
fgr	Growing fraction of viable cells
[Glc]	Glucose concentration (mM)
[Gln]	Glutamine concentration (mM)
[Sug]	Nucleotide as nucleotide sugars lumped concentration (mM)
Kij	Model parameters
μ	Specific growth rate (day ⁻¹)
TCA	Citric acid cycle
UDP-GNAc	Uridine diphosphate <i>N</i> -acetylhexosamine
UDP-GalNAc	Uridine diphosphate <i>N</i> -acetylgalactosamine
UDP-GlcNAc	Uridine diphosphate <i>N</i> -acetylglucosamine
Xd	Specific death rate (day ⁻¹)
Xv	Total viable cell density (10 ⁶ cell/ml)
Qv	Volumetric productivity (mg/l h)

VCH	Volumetric cell-hours (10^6 cell/ml h)
q_p	The specific mAb productivity (1/hr)
GI	Galactosylation Index
SI	Sialylation Index
GI ^{Acc}	Average galactosylation index
GI ^{inst}	Instantaneous GI

Introduction

Glycosylation is a key post-translational process occurring in mammalian cells since it strongly affects the therapeutic properties of mAbs including bioactivity and pharmacokinetics, secretion, solubility, receptor recognition, and antigenicity [26]. The glycosylation process in eukaryotic cells is associated with numerous complex enzymatic reactions whereby sugar groups (oligosaccharides) are attached to the protein's backbone through covalent bonds. The conserved core sugars, which form five out of the fourteen common sugar–lipid-complexes, undergo trimming and branching reactions as the proteins are transported through the endoplasmic reticulum (ER) and Golgi apparatus, resulting in modification of glycans [23]. To achieve therapeutic efficacy, it is crucial to synthesize human mAbs in animal cell cultures so primary structures and post-translational modification are as similar as possible to the naturally occurring ones in the human body. As well, cell culture conditions can also be manipulated to achieve desirable glycosylation patterns [2, 10, 21].

The diversity of glycoforms may manifest itself in site-occupancy (macroheterogeneity) or in the structure of added glycans (microheterogeneity). Macroheterogeneity and microheterogeneity of the glycans can be affected by changing cell culture operating conditions such as nutrients levels, byproduct accumulation, pH and temperature conditions [1, 17]. Temperature has been reported as a highly effective means for increasing the production of mAb [31]. Cellular mechanisms that may explain temperature effects on productivity include increased mRNA stability of recombinant proteins, improved transcription levels, and cell cycle arrest in the G1 phase that leads to energy savings, and reduced consumption of carbon sources towards cell growth [5, 33]. Becerra et al. [3] provided a comprehensive review on the effect of mild hypothermia on cell growth and productivity. According to Becerra et al. [3], mild hypothermia delayed apoptotic pathways by reinforcing cell defense mechanisms, e.g., higher expression of anti-apoptotic proteins. However, the authors propose the occurrence of different cellular mechanisms to explain the possible correlation or lack of correlation between growth and productivity. This correlation may be positive or negative for different cell lines and correspondingly there is no unique mechanism to explain it.

Reducing the culture temperature not only influences cell growth and protein expression but also influences glycosylation [18, 24]. The effects of mild hypothermia on glycosylation appear to be highly dependent on cell line, protein types and temperature, thus explaining some of the contradictory observations reported in the literature. Since the regulation of enzymes during the progress of glycosylation in the ER and Golgi is not well understood, the effect of temperature and the resulting glycosylation has not been fully examined [11, 28]. In early studies by Wang et al. [35] the effect of temperature on glycosylation was explained by changes in residence time of proteins in the Golgi apparatus. Accordingly, lower temperature conditions (21 °C) resulted in decreasing flow rates of glycoproteins through the Golgi with longer exposure of proteins to glycosyltransferases and consequently a higher percentage of *N*-acetyl-lactosamine in the protein structure [35]. In contrast to the aforementioned studies, Hossler et al. reported that sialylation level of EPO-FC decreased by 20 and 40% at 33 and 30 °C, respectively [11]. However, the synergistic effects of low temperature operation (30 °C) and cultivation under 0–5 mM sodium butyrate (NaBu) supplementation did not change the level of total sialic acid content of mAbs produced by CHO cells [6]. It is essential to identify tradeoffs between productivity and glycosylation with respect to culture conditions since very often it is desirable to maximize both. In our previous observations [1], we found higher values of galactosylation and sialylation indices for two culture conditions: (a) Glutamine-free cultures with resulting lower ammonia levels and higher availability of residual Glucose (Glc) and (b) in *Reduced-pH* conditions with resulting lower cell growth and consequent higher residual Glc concentration. For both cases, the productivity was found to be negatively impacted, thus establishing a trade-off between high productivity and high galactosylation levels. In the current study, we are further investigating these tradeoffs for cell cultures operated at lower temperatures.

Mathematical modeling is a suitable way to systematically search for cell culture conditions that result in desired tradeoffs between growth, productivity and glycosylation. A model that correlates the observed mAb's glycan profiles to the cell culture conditions could be useful for optimizing cell culture conditions that will result in desirable glycoprofiles while maintaining high productivity. Since 1996, attempts to model the glycosylation process mostly have considered the reaction rates and folding kinetics [4], the glycosyltransferases kinetics [12], glycosyltransferase kinetics and sugar nucleotide transport rates [7] as the main input of the proposed glycosylation models. However, the number of mathematical models for prediction of glycosylation that can be coupled to the extracellular culture conditions is very limited [25].

The first detailed model of glycosylation was reported by Del Val et al. [7]. This model represented the *N*-glycosylation process within the Golgi apparatus while considering the transport of nucleotide sugar donors from the cytosol to the Golgi lumen, but the model was not explicitly coupled to the extracellular culture conditions. The second model developed by the authors of the current study [21] successfully connected the intracellular glycosylation process to the extracellular culture conditions through a model of nucleotides sugars. Recently Jedrzejewski et al. [13] also extended their mathematical frame work to relate limited cell culture conditions to the glycosylation profiles. However, since both the models of [5] and [13] are based on balances of extracellular species, nucleotides sugars and glycans, they have numerous parameters which make them very challenging to calibrate especially with the limited amount of data. Measurements of intracellular species are particularly scarce since they require large volumes of culture samples for analysis.

Following the above limitations, the current study pursues two objectives: (a) to further study the tradeoffs occurring between mAb productivity and glycosylation levels for changes in temperature of the culture and (b) to develop a simpler semi-empirical model that can describe the effect of extracellular metabolites on the dynamic evolution of glycans. Although the model proposed in the current study is less detailed than the ones reviewed above, it has a much smaller number of parameters as compared to those models and consequently it is expected to be less sensitive to noise and easier to calibrate. This model has been calibrated for different culture conditions including different initial Gln concentration, different temperatures and different pH levels. It is expected that the combination of this model with a dynamic model of

extracellular metabolites could be used in the future for model-based optimization of productivity and glycosylation with respect to cell culture conditions.

Materials and methods

Batch culture

A transfected CHO (DUXB) cell line producing a camelid humanized mAb, Eg2-hFc gratefully received from Yves Durocher, NRC, Montreal [38] was used in the studies. The Eg2-hFc is a smaller size mAb (~80 kD) than a standard human IgG without the heavy chains and it is constructed from a single N-linked glycan at Asn-297. The smaller size of this mAb, its potential longer serum half-life and its distinctive accumulation in tumors make it more attractive for cancer treatment [4].

The CHO (DUXB) cells were cultivated in the BioGro-CHO (BioGro Technologies Inc, Winnipeg, Canada), a serum-free medium for production of Eg2-hFc. Cells were sub-cultured every two days with a seeding density of 0.25×10^6 cells in the BioGro-CHO medium supplemented with 4 mM Gln using 250 mL Polycarbonate shake-flasks. The controlled incubation conditions were 37 °C and 5% CO₂ with a 120 rpm shaking speed (Thermo Scientific, MaxQ shaker). The conditions of the experiments are listed in Table 1.

To investigate the effect of mild hypothermia and initial Gln concentration on cell growth and glycosylation profiles, batch experiments were performed at 25 mM Glucose (Glc) and two levels of Gln (0 and 4 mM), cultures A and B in Table 1 and two levels of temperature (37 and 33 °C) following either one of the two strategies: (a) maintaining the culture temperature at 33 °C from the start of the batch

Table 1 Batch cell culture notifications and experimental designs

Culture notification	Initial concentration (mM)		Cell cultivation conditions		References
	Glucose	Glutamine	pH	Temperature	
A	25	0	No pH adjustment	37 °C	Current Study
B	25	4	No pH adjustment	37 °C	Current Study
C	25	0	No pH adjustment	Shifted Temp from 37 °C to 33 °C on day 3 (Shifted-Temp.)	Current Study
D	25	0	No pH adjustment	33 °C	Current Study
E	25	4	No pH adjustment	Shifted Temp from 37 °C to 33 °C on day 3 (Shifted-Temp.)	Current Study
F	25	4	No pH adjustment	33 °C	Current Study
G	25	8	No pH adjustment	37 °C	[6]
H	25	4	Maintained pH at 6.8 ± 0.05 (Reduced-pH)	37 °C	[6]

operation referred to hereafter as the *Reduced-Temperature* strategy, cultures D and F and (b) shifting the temperature from 37 to 33 °C on day 3 of the exponential phase of growth referred to hereafter as *Shifted-Temperature* strategy, cultures C and E (Table 1). The three days period for the latter strategy was decided based on the assumption that at day three cells are at the end of the exponential phase of growth and consequently a shift in temperature will not affect significantly the overall growth but it may affect glycosylation. Each set of experiments was repeated three times. Two different incubators were operated with two different temperature set points, 33 and 37 °C. Polycarbonate shaker flasks (500 ml) with an initial cell density of 0.2×10^6 cells/ml (cultivated at 4 mM Glutamine and 37 °C) were used for all the batch experiments.

For the purpose of calibrating and testing the model with a richer set of operating conditions, additional experiments were performed at 8 mM initial Gln concentration, culture G. Results obtained in a previous study under lower pH levels to be referred as *reduced-pH* conditions (culture H of Table 1) were also used for testing the proposed model [1]. In the latter case, the average pH of the culture was maintained as low as 6.8 ± 0.05 by daily addition of a determined amount of lactic acid solution. Samples were taken daily for immediate analysis of pH and viable cell density. Additional samples were collected daily, centrifuged at 1000 rpm for 10 min and stored at -20 °C for later glycan and metabolite analysis.

Analytical methods

Cell counting and viability

Cells were counted daily using Trypan blue exclusion method with ZEISS Primo Star microscope with 40× magnitudes. The percentage of viable cells or viability was calculated based on the ratio of viable cells (non-stained) to the total number of cells (dead cells and live cells).

Monoclonal antibody purification and analysis

A Protein A HP spin trap TM column (GE Healthcare 28-9031-32) was used for Eg2-hFc purification prior to the glycan analysis following the manufacturer's instructions.

The enzyme-linked immunosorbent assay (ELISA) method presented in our previous paper was used for monoclonal antibody analysis [1]. The anti-human IgG1 (Fc-specific) (Sigma Aldrich I2136) and the anti-human IgG (Fc-specific)-peroxidase antibody (Sigma Aldrich A0170), both produced in goats, were used as the primary and the secondary antibodies, respectively. The 3, 3', 5, 5'-tetramethylbenzidine dihydrochloride (Sigma Aldrich T3405) was used as the substrate for peroxidase. The optical

density of the developed color was determined at 450 nm with a micro-plate reader (Synergy 4, BioTek) after 30 min at room temperature (25 °C) incubation. The optical density of each sample was compared with a pre-determined standard curve of concentrated EG2-hFc and final mAb concentration was calculated.

Glucose, lactate, ammonia and amino acids concentrations

A multi-parameter bioanalytical system, Bioprofile 400 (Nova Biomedical, Waltham, MA, USA), was used for determining glucose, glutamine, glutamate, lactate and ammonia concentrations.

Glycan analysis

The relative abundances (RA) of glycoforms of EG2-hFc (including both fucosylated and nonfucosylated glycans) were identified using a recently developed method [30]. Briefly, mAbs were captured and purified in a series of mini protein A, HP SpinTrap columns (GE Healthcare Life Sciences, Cat. # 28-9031-32) using a phosphate buffer solution as wash buffer. Then, glycan content of samples was released from the mAb bound in the mini protein A columns using 10–20 units of peptide-*N*-glycosidase F (Promega Corporation, Cat. # V4831) with a 24 h incubation at 37 °C on a rocker shaker. Then, glycans were eluted using phosphate buffer (Sigma Aldrich, Cat. # 47877) and dried in a vacuum concentrator and labeled using 2-aminobenzamide (2-AB). Hydrophilic interaction liquid chromatography (HILIC) was used to determine Glc unit (GU) values of the glycans by comparing them to a 2-AB-dextran ladder as previously described [1]. The glycan structures were initially identified based on the GU values reported in the Glycobase database (<https://glycobase.nibrt.ie>). Exoglycosidase enzyme array digestion was used as explained in an earlier study [26] for confirmation of the identified structures using Sialidase A (recombinant from *A. ureafaciens*), bovine kidney α -fucosidase, bovine testis β -galactosidase, Jack Bean β -N-acetylhexoaminidase (Prozyme). Digested glycans were re-analyzed using HILIC analysis and shifts of glycan GU values were compared (Fig. S1).

Glycan structures and glycosylation indices

The main glycan structures observed for EG2-hFc were complex biantennary fucosylated types with one or two galactoses followed by one or two sialic acids. Regarding occupancy of available positions in the biantennary structure G_i^1 (monogalactosylated), S_i^1 (monosialylated), G_i^2 (digalactosylated), S_i^2 (disialylated), G_i^0 (agalactosylated) and S_i^0 (asialylated) were identified. The summary of glycan structures is provided in Table S.1 of supplementary

materials. To simplify the quantification of the evolution of glycan structures during the course of a culture at different operational conditions, glycosylation and sialylation indices were calculated [1, 21] according to the following equations:

$$GI = \frac{\sum G_i^2 + 0.5 \times \sum G_i^1}{\sum G_i^2 + \sum G_i^1 + \sum G_i^0} \quad (1)$$

$$SI = \frac{\sum S_i^2 + 0.5 \times \sum S_i^1}{\sum S_i^2 + \sum S_i^1 + \sum S_i^0} \quad (2)$$

Specific metabolites' production and consumption rates

The specific mAb productivity (q_p) and production or consumption rates of metabolites (q_{Met}) such as glucose, glutamine, lactate, and ammonia were calculated from the slope of the regressed lines of the cumulative mAb (C_{mAb}) or metabolite concentrations (C_{Met}) against the cumulative volumetric cell hours (VCH) as given by Eqs. 3 and 4. Equation 3 describes volumetric cell hours that is a measure of the cumulative time (t) spent by all viable cells (X_v) in the culture as proposed by Dutton et al. [8]. The maximum volumetric productivity ($q_{v,max}$) of mAbs was calculated based on the equation presented by Yoon et al. [37] by dividing the maximum mAb concentrations by the corresponding culture time (Eq. 5). The specific productivity can be obtained from the slope of the plot of mAb as a function of VCH.

$$VCH = \sum \frac{X_v(i+1) - X_v(i)}{\ln(X_v(i+1)/X_v(i))} (\Delta t) \quad (3)$$

$$q_{p/Met} = C_{Mab/Met} / VCH \quad (4)$$

$$q_{v,max} = C_{Mab} / \text{time} \quad (5)$$

Semi-empirical glycosylation model

A semi-empirical mathematical model is proposed to correlate the extracellular culture conditions to the resulting galactosylation of the antibody. The preliminary objective of this model was to elucidate correlations between culture conditions to the GI evolution in the course of culture. This simplified model could be also used in the future in combination with a model of extracellular metabolites to optimize the process in terms of productivity and galactosylation but this objective is beyond the scope of this study.

A rigorous model of glycosylation has been previously proposed by the co-authors. This rigorous dynamic model consisted of three main sub-models: (a) a metabolic flux

analysis (MFA)-based dynamic model, (b) a nucleotide sugars model and (c) a glycosylation model in the Golgi apparatus [21]. The predicted results of temporal glycosylation indices were in acceptable agreement with the experimental glycosylation indices of mAbs produced by CHO cells under batch condition with two initial Gln concentrations. However, this comprehensive model is of very high dimensions involving over 100 differential equations and a large number of parameters, thus making it difficult to calibrate for a wide range of operating conditions. Also, providing adequate nucleotide sugars and glycan data at a variety of culture conditions with good repeatability is challenging. To circumvent the problems associated with our earlier mechanistic model, we propose in this study a semi-empirical model that can correlate the extracellular culture conditions and glycosylation while avoiding the very challenging parameter estimation task required for a more rigorous model.

The proposed semi-empirical model consists of three parts:

- A dynamic balance of a lumped quantity representing the total nucleotide sugar intracellular concentration (Eq. 6)
- A static correlation describing the galactosylation index as a function of parameters that are expected to affect this index such as the nucleotides sugars levels, the specific antibody productivity through its effect on residence time in the Golgi and the Glc levels (Eq. 7) and
- An equation describing the cumulative average of the GI values to account for the accumulation of antibodies with different galactosylation levels over the course of the cell culture (Eq. 8).

$$\frac{dSug}{dt} = a_1 \mu(t)^m \frac{[Glc]^p}{a_2 + [Glc]} (1 + [Gln])^q - a_3 ([Sug]) \quad (6)$$

$$GI^{inst} = (1 + a_7) \mu(t)^n \frac{1}{(q_p(t) + a_4)^r} \left(\frac{[Glc][Sug]}{(a_5 + [Glc])(a_6 + [Sug])} \right) \quad (7)$$

$$[GI]^{Acc} = \frac{\int_0^t \Delta mAb [GI]^{inst} dt}{\int_0^t \Delta mAb dt} \quad (8)$$

where [Sug] is the lumped concentration of all types of nucleotide as nucleotide sugars present within viable cells at one-day intervals. GI^{inst} represents an instantaneous galactosylation index resulting from the galactosylation

process occurring within the Golgi at a particular time interval. Equation (7) predicts the GI value for a specific time instance as a function of specific growth rate (μ), specific mAb productivity (q_p), Glc concentration [Glc], and lumped concentration of all types of nucleotide as nucleotide sugars [Sug]. As this equation does not consider the accumulation of glycan concentration along the culture cultivation, the average galactosylation index of the mAb (GI^{Acc}) present in the batch culture at any given time requires the calculation of a cumulative average based on the mAb productivity (ΔmAb) and the instantaneous GI values ($[GI]^{inst}$) given by Eq. 8.

Results and discussion

Effect of culture conditions on cell growth, extracellular metabolites and mAb productivity

As mentioned in the introduction, the effect of hypothermia on growth and productivity has been studied before while the effect of hypothermia on glycosylation has not been thoroughly understood and the limited studies available had reported contradictory observations. In this section, we briefly discuss the effect of hypothermia in combination with two different initial Gln concentrations on growth and productivity with the goal to relate these

findings to glycosylation. According to earlier studies [1], cell growth for this cell line was maximal at 4 mM of Gln and 25 mM of Glc. On the other hand, we found that cultures initiated without Gln and with 25 mM of Glc had glycosylation indices (GI and SI) that were significantly higher as compared to cultures supplemented with Gln. Thus, general tradeoffs were observed between growth and galactosylation, i.e., higher growth often resulted in lower galactosylation index. In view of these competing outcomes, temperature manipulation was tested for only these two sets of conditions, i.e., 0 mM Gln and 25 mM Glc and 4 mM Gln and 25 mM Glc, to assess whether it is possible to alter the previously identified tradeoffs between productivity and glycosylation.

The temperature adjustments were applied as per the strategies explained in Table 1 of the materials and method section (Sect. 2.1).

The cell density profile and viability indices for cultures cultivated at either 4 mM Gln or 0 mM Gln in combination with the aforementioned mild hypothermia strategies (cultures C, D, E and F of Table 1) are compared with a corresponding control (cultures A and B of Table 1) in Figs. 1 and 2. Comparing the results of temperature effect with and without initial supplementation of Gln shown in Figs. 1 and 2, it can be seen that the main effect of mild hypothermia was a general reduction of the specific growth rates and an extension of the cell viability.

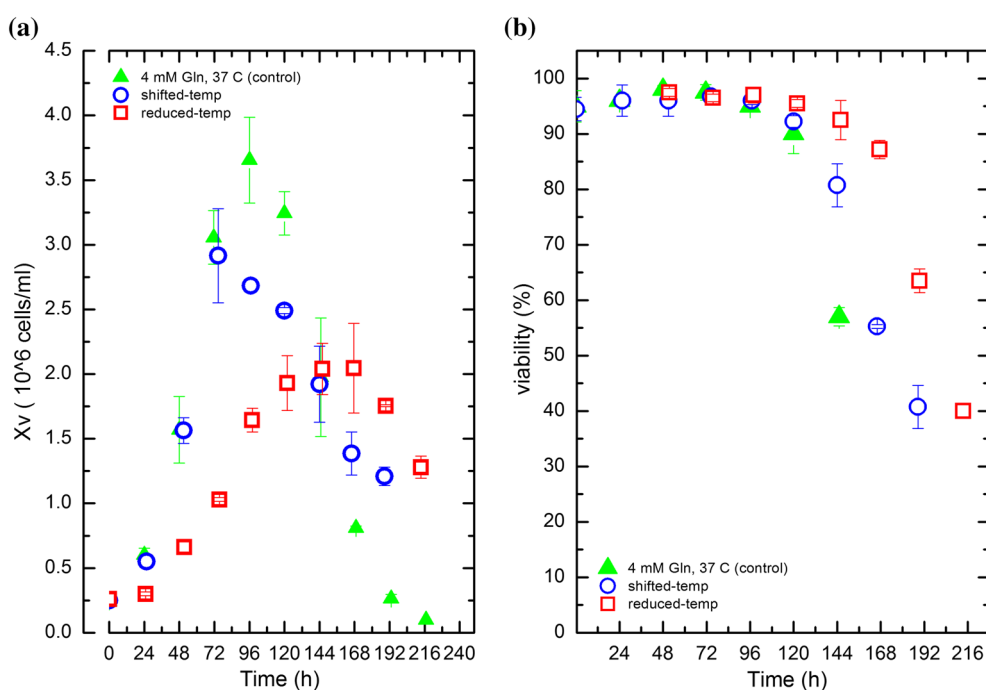


Fig. 1 Time profile of total viable cell density (a) and viability (%) (b) of CHO cells cultivated at 4 mM Gln and different temperature conditions. Data points represent mean $\pm$ SD for triplicate experi-

ments. Filled triangle control at 37 °C, open circle shifted-temperature and, open square reduced-temperature

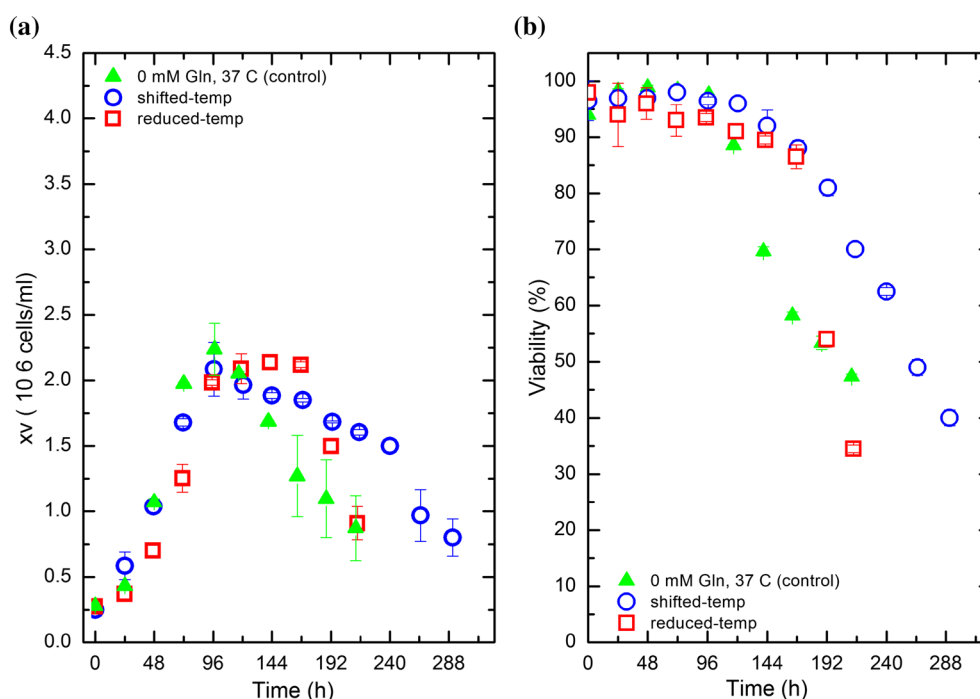


Fig. 2 Time profile of total viable cell density **(a)** and viability (%) **(b)** of CHO cells cultivated at 0 mM Gln and different temperature conditions. Data points represent mean $\pm$ SD for triplicated experi-

ments. Filled triangle control (37 °C), open circle shifted-temperature and, open square reduced-temperature

The reduction in specific cell growth under reduced temperature conditions is in agreement with previous studies [18, 33]. However, in the current study it was also observed that for cultures initially supplemented with Gln and the *Reduced-Temperature* regime, culture F, the viable cell density could not reach the same level as the control condition (culture B), even at the later stages of cultivation in contrast with previously reported results [29]. The observed prolonged period of high cell viability under the lower temperature conditions is in agreement with other studies [18, 19].

The extracellular concentrations of glutamine, glucose, lactate and ammonia for batches of 4 mM or 0 mM initial Gln concentrations are presented in Figs. 3 and 4, respectively. It is evident from these figures that for both batches initiated with 4 mM and 0 mM Gln (cultures C, D, E and F of Table 1), the concentrations of Glc and Gln at the end of the batches were higher for both the *Shifted-Temperature* and *Reduced-Temperature* samples as compared to the control (cultures A and B of Table 1). For example, for 4 mM initial Gln the control culture consumed all the Glc by day 5, whereas for the *Shifted-Temperature* and *Reduced-Temperature* sufficient amounts of Glc (3.3 ± 0.4 and 6.6 ± 0.8 mM, respectively) were left in the culture for sustaining further growth. Gln was consumed even faster than glucose. This fact can be explained by the lower cell growth observed under mild hypothermia conditions, which led to

further preservation of Glc and Gln for a longer duration in the culture.

Lactate profiles were very similar for all the cultures during the exponential phase. Lactate was produced at a higher rate during the exponential phase of growth and then consumed to a slight extent during the post-exponential phase.

For both 4 and 0 mM initial Gln levels (cultures A and B of Table 1), lactate consumption was lower at the lower temperature experiments due to the availability of Glc towards the end of the batches. Specific ammonia accumulation was higher in the exponential phase of growth for all conditions and significantly decreased in the decline phase. The rate of ammonia formation for the experiments with initial 0 mM Gln (cultures A, C and D of Table 1) was lower as compared to the batches initiated with 4 mM initial Gln concentration due to the expected lower production of ammonia from glutamine and also to the utilization of ammonia for Gln synthesis (cultures B, E and F of Table 1) [34].

The mAb concentration profiles as a function of time or as a function of VCH (volumetric cell hours) for cultures cultivated with 4 mM and 0 mM initial Gln levels under mild hypothermia (cultures C, D, E and F of Table 1) and control conditions (cultures A and B of Table 1) are presented in Fig. 5a, b, respectively. The specific productivities were calculated from the slopes to the evolution of mAb

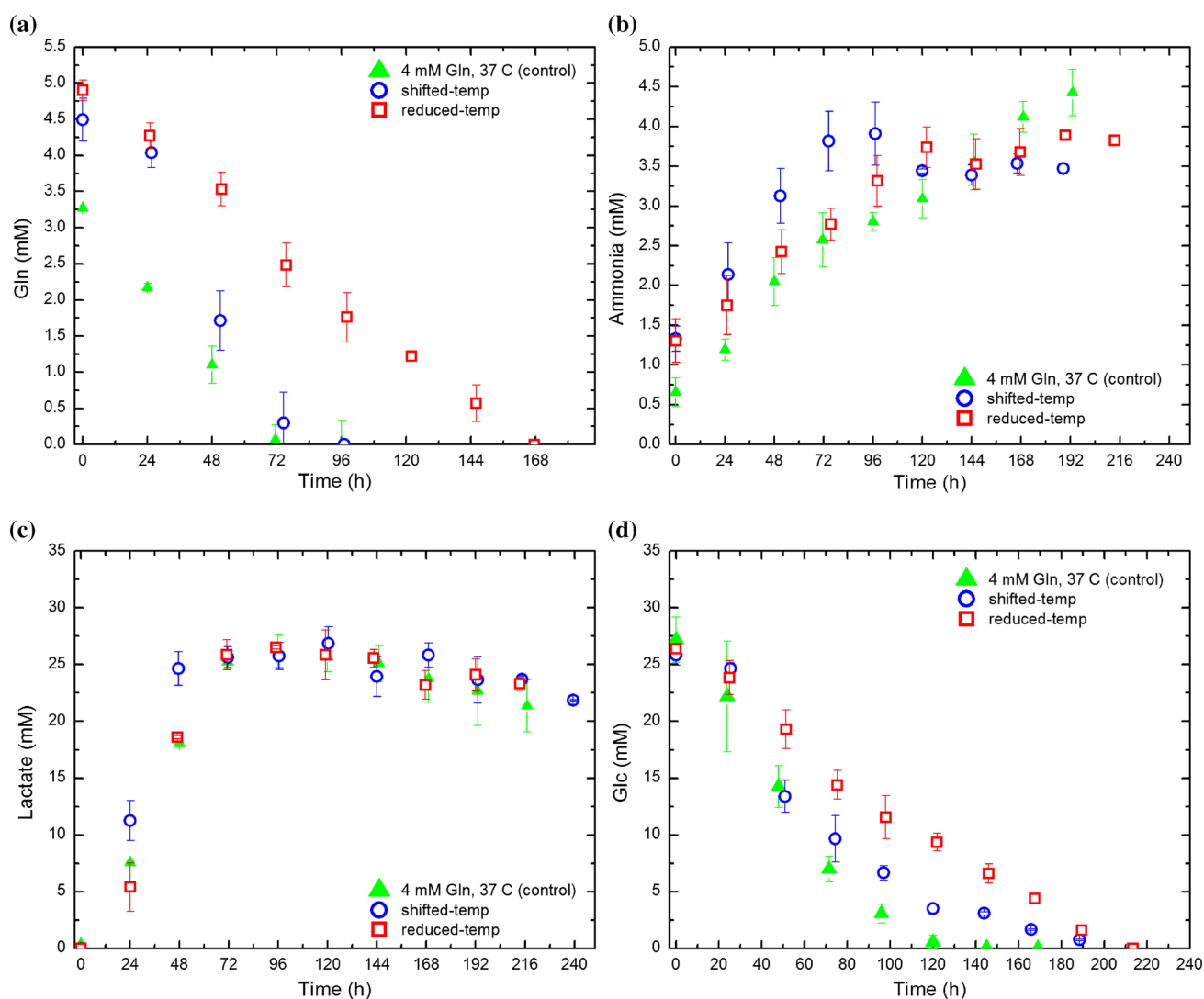


Fig. 3 Metabolite profiles of mild hypothermia experiment at 4 mM Gln. Data points represent mean $\pm$ SD for triplicate experiments: Filled triangle control at 37 °C, open circle shifted-temperature, and

open square reduced-temperature. **a** Gln (mM), **b** ammonia, **c** lactate (mM), **d** Glc (mM)

with respect to VCH. For both 0 mM and 4 mM initial Gln levels, the concentration of mAb monotonically increases under *Reduced-Temperature* conditions throughout the cultures. In the 4 mM initial Gln cultures, the productivity decreased during the post-exponential phase of growth for both the control and the *Shifted-Temperature*. For the 0 mM initial Gln case, the productivity decreased in the post-exponential phase but increased in the *Shifted-Temperature* culture. Thus, shifting the temperature at the post-exponential phase of growth was more effective in increasing mAb productivity for the culture initiated with 0 mM Gln as compared to the 4 mM initial Gln case.

Table 2 summarizes the specific productivities during the exponential and post-exponential phases. For example, the results indicate that for 4 mM initial Gln reducing

temperature for the entire duration of the culture (9 days) can improve q_p up to 25% for the exponential phase (culture F of Table 1); and up to a threefold increase for the post-exponential phase of growth as compared to the control batch (culture B of Table 1). In terms of productivity, reducing the temperature towards the end of the exponential phase of growth was not as effective as reducing the temperature for the entire culture time. However, even for the *Shifted-Temperature* experiment (culture E of Table 1), q_p was increased in the post-exponential phase of growth as compared to the control experiments.

The maximum volumetric productivity rates, $q_{v,max}$, calculated from Eq. 5, are shown in the supplementary material (Fig. S.2). For initial 4 mM Gln, the value of $q_{v,max}$ was not significantly increased by shifting temperature to 33 °C at

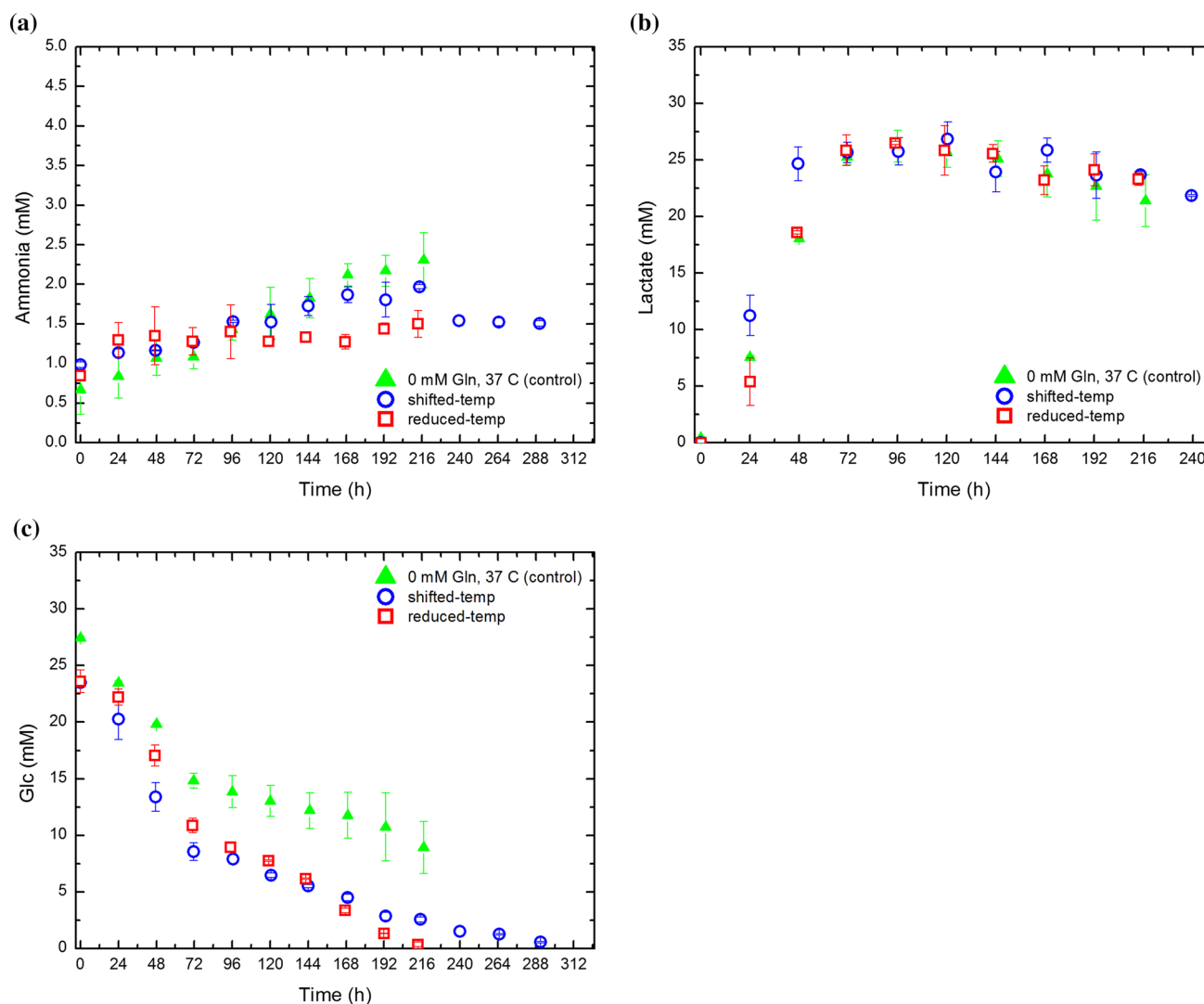


Fig. 4 Metabolite profiles of mild hypothermia experiment at 0 mM Gln. Data points represent mean $\pm$ SD for triplicate experiments: *filled triangle* control at 37 °C, *open circle* Shifted-Temperature, and *open square* Reduced-Temperature. **a** Ammonia (mM), **b** lactate (mM), **c** Glc (mM)

day three (culture E of Table 1) as compared to the control (culture B of Table 1) but under *Reduced-Temperature* conditions, the maximum volumetric productivity increased by over 100% as compared to the control. On the other hand, for 0 mM initial Gln the maximum volumetric productivity, $q_{v,max}$ (Fig. S.2) significantly increased for both *Shifted-Temperature* (2 fold) and *Reduced-Temperature* (2.5 fold) cultures (cultures C and D of Table 1) as compared to the control (culture A of Table 1). The improved final mAb concentration in the case of initial 0 mM Gln was not related to the observed extended culture longevity, but rather to a marked increase of specific mAb productivity. Increased mRNA stability of recombinant proteins at lower temperature and improved transcription levels have been reported as possible mechanisms for increased productivity. On the other hand, we did not observe any reduction in metabolism

of carbon sources by reducing temperature as reported by other researchers [27, 33].

N-linked glycosylation pattern and glycosylation indices

Since the time evolutions in glycosylation for 0 and 4 mM initial Gln concentrations were significantly different, they are discussed below in two separate subsections.

Combination of mild hypothermia and 4 mM initial Gln

The EG2-hFc mAb has predominantly fucosylated biantennary glycans, specifically F6A2G0, F6A2G1, F6A2G2, F6A2G2S1 and F6A2G2S2. Differences between glycosylation of the *Shifted-Temperature* and *Reduced-Temperature* cultures as compared to the control experiment

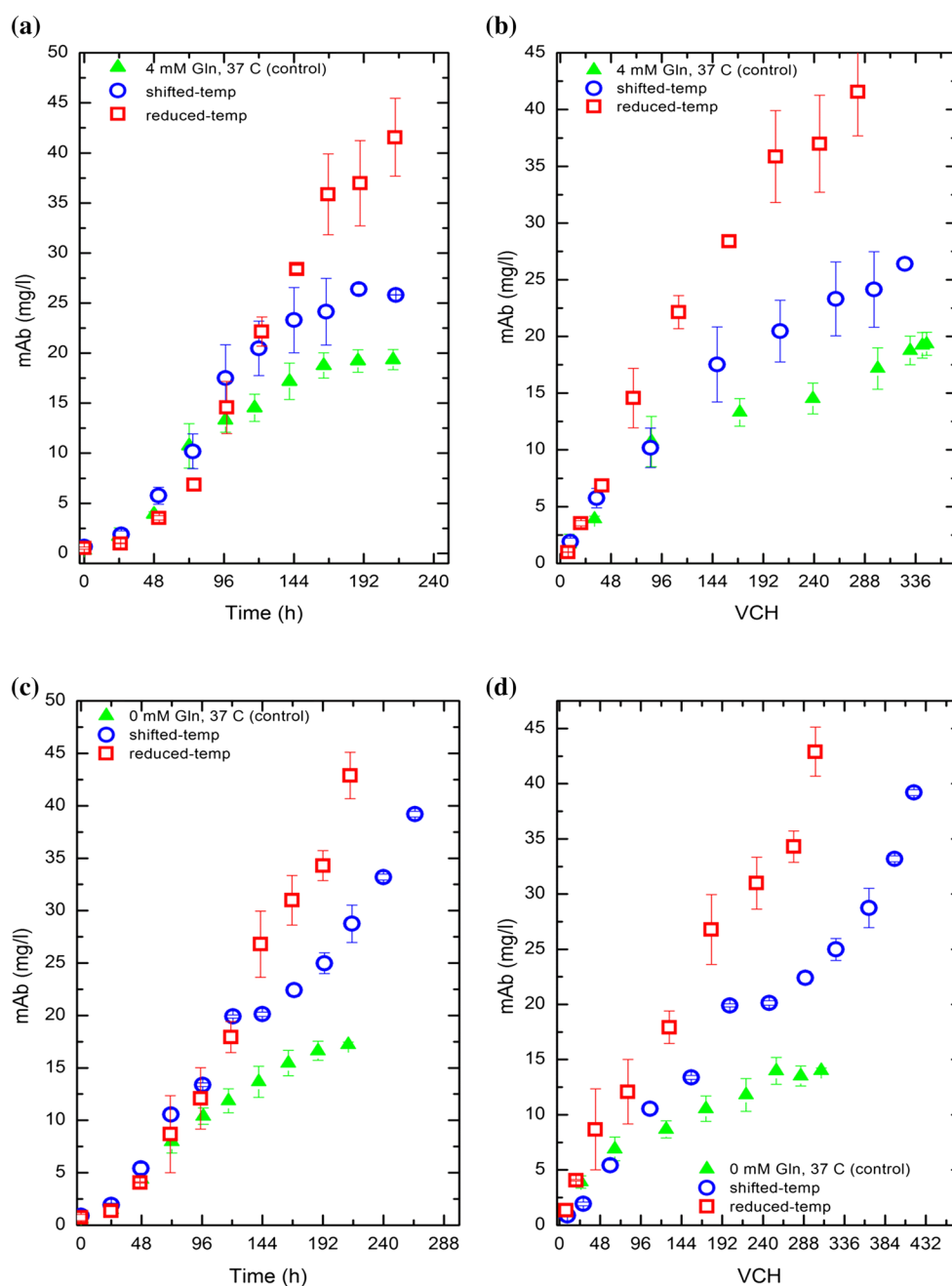


Fig. 5 mAb profiles and mAb vs VCH (Eq. 4) of mild hypothermia experiment at 4 mM Gln (a) at 0 mM Gln (b). Data points represent mean $\pm$ SD for triplicate experiments: *filled triangle* control at 37 °C, *open circle* shifted-temperature and, *open square* reduced-temperature

Table 2 Specific mAb productivity under mild hypothermia conditions at exponential and post-exponential phase of growth

Gln	Specific mAb productivity q_p (10^{-12} g/cell h)					
	Exponential phase of growth			Post-exponential phase of growth		
	Control (37 °C)	Shifted-temp.	Reduced-temp.	Control (37 °C)	Shifted-temp.	Reduced-temp.
4 mM	0.120	0.083	0.150	0.036	0.054	0.150
0 mM	0.120	0.120	0.130	0.039	0.078	0.130

were observed (Tabel S.2). For example, on day 3, F(6) A2G0, a non-galactosylated glycan reached a two-fold higher percentage area of 14.11 ± 2.24 under the *Reduced-Temperature* operation (culture F of Table 1), as compared to the *Shifted-Temperature* (7.06 ± 0.37) and control (6.24 ± 0.3) experiments (cultures E and B of Table 1). On the other hand, under *Shifted-Temperature* and control operations, the level of this glycan consistently increased until the end of culturing to the value of 25.13 ± 1.56 and $17.12 \pm 0.85\%$, respectively, while at *Reduced-Temperature* batches its value drops by day 5 of the culture to the value of 8.3 ± 0.23 . The lower availability of Glc observed towards the end of the cultures in the control and the *Shifted-Temperature* batches as compared to the *Reduced-Temperature* batches may explain these trends [17, 21].

On the other hand, the maximum levels of F(6)A2G2, a fully galactosylated glycan, reach on day 3 for the *Shifted-Temperature* regime (culture E of Table 1) and for the control batch (culture B of Table 1) values of 45.87 ± 1.5 and $46.47 \pm 2.3\%$, respectively, while for the *Reduced-Temperature* (culture F of Table 1) this maximum was delayed to day 4, with a value of $40.09 \pm 0.77\%$. According to Eq. (1) for GI (galactosylation index), the relevant abundance (RA) of this glycan only appears on the numerator and as its value gets smaller, the GI value drops toward the end of the culture. Similar to galactosylation, at *Reduced-Temperature* conditions the maximum in the sialylation index occurs at a later day of culture compared to the *Shifted-Temperature* and the control batches. The highest levels of sialylated glycans mainly, F(6)A2G2S1 and F(6)

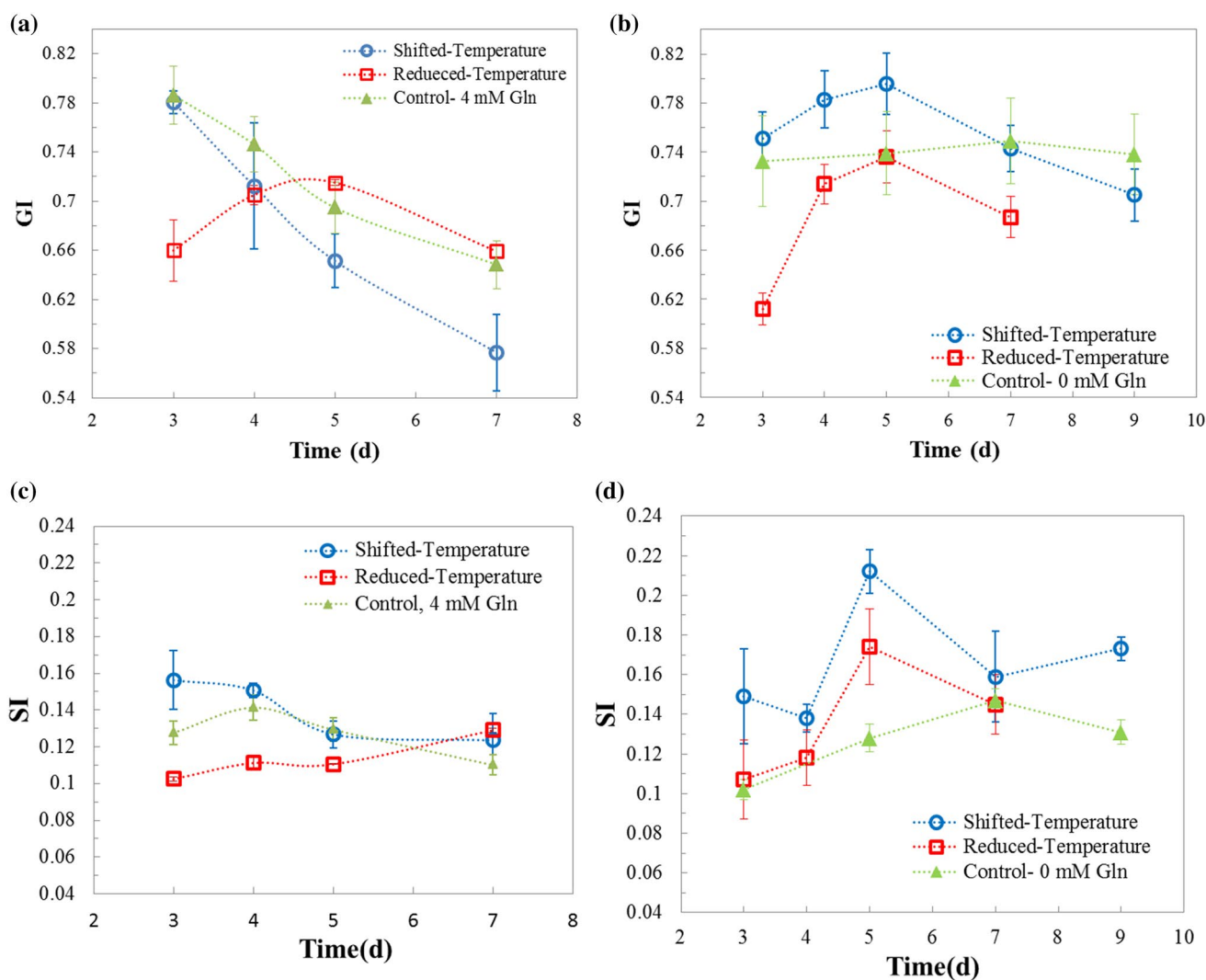


Fig. 6 Galactosylation index (GI) profile (a) at 4 mM Gln and hypothermia conditions, b at 0 mM Gln and hypothermia conditions. Sialylation index (SI) profile, c 4 mM Gln and hypothermia conditions,

d 4 mM Gln and hypothermia conditions. Data points represent mean for duplicated experiments: filled triangle control at 37 °C, open circle shifted-temperature, and open square reduced-temperature

A2G2S2 were $13.02 \pm 0.42\%$ and $3.64 \pm 1.03\%$ on day 3 of the *Shifted-Temperature* culture, and 12.31 ± 0.61 and 2.27 ± 0.2 for the control batch. In contrast, the maximum value of F(6)A2G2S1 and F(6)A2G2S2 for the *Reduced-Temperature* flasks happens on day 5 and day 7 with the value of $7.26 \pm 0.06\%$ and $1.93 \pm 0.01\%$, respectively. We have previously reported that ammonia inversely affects the sialylation index [1]. However, since mild hypothermia did not alter the ammonia production rate and approximately the same concentration of ammonia of 4 ± 0.3 mM was obtained for all the three conditions ammonia production cannot explain this delayed sialylation. Instead it is hypothesized that correlation between galactosylation and sialylation, as sialic acid can only attach to a galactose branch of a glycoprotein, could be the main cause of delayed sialylation in *Reduced-Temperature* batches [1, 17, 21].

The time profiles of the glycosylation indices are shown in Fig. 6 A and 6 B. These values were calculated according to Eqs. 1 and 2 based on the relevance abundance (RA %) values presented in Table S.2. Glycan analysis was available only after day 3 of each culture since this analysis required a certain minimal amount of mAb that was not available earlier in the culture. Then, in order to assess the evolution of glycans during the first days of the culture, we assumed that at time = 0 GI was equal for all the batches to the observed values in day 3 of the control culture (GI = 0.78, Fig. 6a). This assumption was based on the fact that all experiments were started with an inoculum collected from a previous culture conducted for 3 days at control conditions (culture B of Table 1).

Under the control and the *Shifted-Temperature* conditions (cultures B and E of Table 1), the measured galactosylation index, GI value, Fig. 6a) exhibited the largest level on day 3 as compared to the values measured in subsequent days, and its profile declined approximately by 19%, for the control and 26% for the *Shifted-Temperature* between day 3 to day 7. Significantly different trends of the GI profile were observed for the *Reduced-Temperature* condition (culture F of Table 1). For example, the GI at day 3 was 0.66 thus indicating a possible initial decrease from the assumed initial value of 0.78 where the rationale for this assumption was given above. This observed value of GI at day 3 was much lower than the values observed for the *Shifted-Temperature* and control cultures (~ 0.78). The highest measured level of GI for *Reduced-Temperature* conditions occurred on day 5 and it was still lower than the maximum GI values for the control and *Shifted-Temperature* conditions (Fig. 6a). The possible reasons for the reduced glycosylation at the beginning of the *Reduced-Temperature* cultures are increased productivity [20], lower cell growth associated with lower synthesis of nucleotides

and lower synthesis rate of glycans [24], lower expression of glycosyltransferases at lower temperature or decrease of nucleosides at low temperature [14]. The mathematical model presented in Sect. 3.2 was used to elucidate these effects revealing that the growth is strongly correlated to GI but specific productivity may not have a significant effect on GI changes. The subsequent drastic decrease in galactosylation occurring between days 5 and 7 for both the *Shifted-Temperature* and *Reduced-Temperature* operations is correlated to the depletion of Glc occurring during this period as shown in Fig. 3d.

As shown in and Fig. 6a, c, the galactosylation index (GI) and sialylation index (SI) are correlated [4]. Accordingly, the SI value at days 3, 4 and 5 for the *Reduced-Temperature* culture was also lower with respect to the *Shifted-Temperature* and the control conditions (Fig. 6c) and, similarly to the GI, the SI value increased between day 3 and day 7 for the *Reduced-Temperature* operation. In summary, hypothermia applied during the entire culture was favorable for mAb production, but overall it reduced the final galactosylation and sialylation levels.

Combination of mild hypothermia and 0 mM initial Gln

F(6)A2G0, the dominant non-galactosylated glycan (Table S.3), on day 3 reached a higher level of $14.5 \pm 1.56\%$ under the *Reduced-Temperature* regime (culture D of Table 1) as compared to the *Shifted-Temperature* ($8.77 \pm 3.39\%$) and Control (7.28 ± 0.36) operations (cultures C and A of Table 1). Subsequently, the levels of this glycan declined to 8.8 ± 1.3 and $6.14 \pm 0.17\%$ on days 4 and 5, respectively, for the *Reduced-Temperature* condition, and again rose to $12.73 \pm 0.83\%$ on day 7. For the *Shifted-Temperature* operation, the profile of F(6)A2G0 for the cultures initiated without Gln was different from the profiles obtained in cultures initiated with 4 mM Gln since its relative abundance did not increase monotonically after day 3. The profiles of the fully galactosylated glycan F(6)A2G2 also exhibited differences for cultures started without glutamine supplementation compared to 4 mM Gln batches (Table S.3). Without glutamine, the F(6)A2G2 reaches its higher value on day 4 for all the conditions of *Shifted-Temperature*, *Reduced-Temperature* and Control (45.7, 39.75, and 41.97%, respectively) and its value remained high up to day 5 with a subsequent significant drop on day 7. This trend is similar to that observed for the 4 mM Gln and *Reduced-Temperature* batch but different from the trends observed for 4 mM Glutamine and *Shifted-Temperature* and 4 mM Gln (control) experiments. The observed high galactosylation levels appear to be correlated to higher availability of residual glucose for cells when either cultivated with glutamine supplementation at *Reduced-Temperature* (Fig. 3d)

or without initial glutamine addition regardless of the temperature conditions (Fig. 4c).

To further understand the effect of temperature on the overall glycan evolution, the GI (galactosylation index) and SI (sialylation index) were calculated (Fig. 6). Similar to the 4 mM initial Gln experiments, for the combined *Reduced-Temperature* and zero initial Gln experiment, the GI profile was different from the control. On day 3, a lower level of GI equal to 0.61 was observed compared to 0.75 and 0.74 GI values for the *Shifted-Temperature* and the control batches, respectively. Then, the level of GI increased to a value of 0.72 on day 5, very close to the value observed for the control batch. The low level of GI on day 3 for the *Reduced-Temperature* batch can be again explained by similar reasons as listed for the 4 mM culture. In clear contrast, the GI in the control culture (0 mM Gln, 25 mM Glc and 37 °C) remained almost constant during the course of the batch possibly because sufficient Glc was available until the end of cultures (9 mM of Glc on day 9) as compared to the *Shifted-Temperature* and *Reduced Temperature* that ended with significantly lower concentrations of Glc (0.6 and 0.3 mM, respectively).

Reducing the temperature, for both the *Shifted-Temperature* and *Reduced-Temperature* operations increased sialylation levels specifically on the day 5 of cultivation.

Again, this trend can be explained by the correlation between GI and SI, as the value of GI also reached a maximum on day 5.

In summary, our results revealed that reducing the temperature with respect to control conditions (37 °C) reduces galactosylation. The correlation between temperature reduction strategies and galactosylation is further discussed in the mathematical modeling section that follows.

Modeling results

A semi-empirical glycosylation model was developed to explain the experimentally observed correlations between extracellular culture conditions and the level of mAb galactosylation. The aim was to develop a simple model with few calibration parameters [13, 21] that would be applicable for a range of operating conditions (Table 1) in terms of different initial Gln concentrations (cultures A, B and G) [1], temperatures (cultures C, D, E and F) and lower pH conditions (culture H) [1].

For the calibration of this model, we hypothesized dependencies of Eqs. (6) and (7) with respect to different culture (Table 1) parameters based on knowledge from the literature and the comprehensive model of [21] and then these dependencies were either confirmed or invalidated based on the ability of the model to fit the data. To calibrate the model data collected under different *Reduced-Temperature* conditions (cultures D and F of Table 1), their

corresponding controls (cultures A and B of Table 1) were used. In addition, data from a previous study obtained with 8 mM initial glutamine (culture G of Table 1) and with reduced pH levels (culture H of Table 1) were also considered to expand the validity of the model. Then, the prediction accuracy of the model was tested for *Shifted-Temperature* conditions (cultures C and E of Table 1) that were not used for model calibration.

For the nucleotide sugars' model, Eq. 6, it was assumed that the concentration of these sugars is a function of extracellular Glc and Gln concentrations based on the full mechanistic model reported by [15], and the reported effects of glucose on the nucleotide sugar pools [16, 32, 36]. In addition, it has been found that for the CHO cell line under study the consumptions of Glc and Gln are strongly coupled [1, 21] and, therefore, they have been both included as multiplying factors in the numerator of Eq. (6). The hypothesized dependency of the nucleotides sugars synthesis term with respect to growth rate was based on the fact that these sugars are synthesized from nucleotides which serve as substrates for nucleic acids' that are needed for biomass formation [13].

Equation 7 is solved in combination to Eq. 6 to calculate the GI. In (7) GI is assumed to be dependent on the specific mAb production rates, extracellular Glc concentrations and cell growth. The dependency of galactosylation on nucleotide sugar is evident since the latter are substrates for all the glycosylation reactions [16, 21, 36]. The assumed effect of specific productivity on galactosylation is based on the fact that the mAb productivity may affect the residence time of proteins within the Golgi, thus determining the extent of glycosylation [35]. A long residence time was expected to result in a high glycosylation. However, the effect of specific productivity on residence time in the Golgi is controversial since some researchers argue that as productivity increases the volume of the Golgi to accommodate the higher production of proteins [12]. The additional variable considered in Eq. (7) is [Glc]. In our earlier study, we have demonstrated that higher availability of Glc at later stages of the batch resulted in further galactosylation and sialylation [1]. The Glc availability also has been reported to affect the site occupancy or macroheterogeneity of the glycan profile through alteration of lipid-linked oligosaccharides [17, 22]. As Glc itself is involved as a substrate in the synthesis of nucleotide and nucleotide sugars, a Michaelis–Menten expression involving Glc was assumed for this reaction. The assumed dependency of the GI^{inst} term on growth rate is based on the fact that the pool of nucleotide and nucleotide sugars serves both as substrates for galactosylation and for biomass formation [13].

As mentioned, Eq. 7 depicts the instantaneous GI level in one individual cell while the GI measurement of a supernatant sample will reflect a cumulative value of

the glycosylated mAbs cultivated until the time of sample analysis. To convert the instantaneous value into an average over culture time, Eq. 8 is used where a Δ mAb amount of mAb is produced during a one-day interval assuming that the glycoprofile remains constant during that day.

The parameters of Eqs. 6 and 7 were optimized by minimizing the sum of squared error (SSE) between experimental (Table 1) and model predicted results using *fmincon* solver in MATLAB environment. The initial value for GI at time = 0 was assumed to be equal to the GI at day 3 of the control culture with 4 mM initial Gln (4 mM Gln, 25 mM Glc and 37 °C). As explained in the previous section, this is based on the fact that all the cultures were started with an inoculum obtained from a previous culture conducted for only 3 days at these experimental conditions. Many different structures of the model were tried and the model shown in Eqs. (6–8) is the one resulting in the lowest sum of square errors between predictions and data. Also, since the calibration involves a nonlinear optimization, we tried a large number of initial guesses to better approximate a global optimum. All parameters were assumed positive and optimized within pre-specified boundaries. The optimized parameter values are provided in the Table S.4. Figures 7 and 8 present the value

of GI predicted by the index model in comparison with the experimental data obtained at a variety of experimental conditions shown in Table 1. It is interesting that the parameters q and m that reflect the effect of Gln and specific growth rate, respectively, converged to their lower boundaries of zero, thus indicating weak effects of Gln on nucleotide sugars and specific cell growth on GI. Specifically, the synthesis of sugars described by Eq. (6) is mainly correlated to glucose. On the other hand, the GI described by Eq. (7) is correlated to the nucleotides sugars levels calculated from (6), to the growth rate, to glucose concentration and in a lesser degree to the inverse of the specific productivity. Thus, the observed effects of temperature on galactosylation are mostly captured through the effect of temperature on growth rate as in Eq. (7) where this effect can be explained by a reduced expression of glycosyltransferases as reported in [24].

In general, the developed model was able to successfully capture the galactosylation index trends during the course of the culture at different cell culture conditions (Table 1). For example, the model correctly captures the initial larger decrease in GI for the cultures operated under mild hypothermia (Figs. 7d, f, 8c) as compared to the ones conducted at 37 °C (Fig. 7a, b) and the smaller GI in the

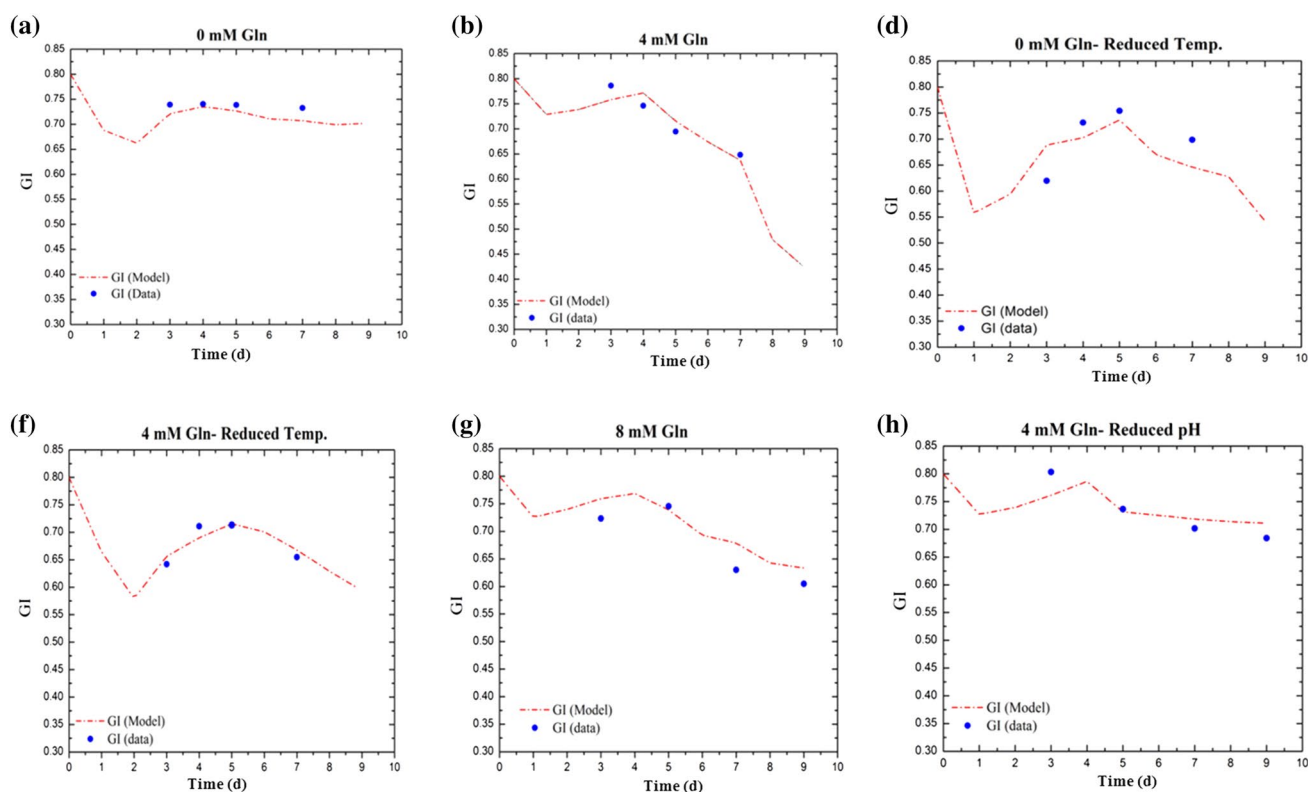


Fig. 7 Profile of experimental and predicted GI at different culture conditions for model calibration. Data points present experimental data and *dash lines* display simulated data as described in Table 1. R^2 value calculated as 0.80. **a** 0 mM Gln at 37 °C, **b** 4 mM Gln at 37 °C,

d 0 mM Gln at Reduced-Temperature of 33 °C, **f** 4 mM Gln at Reduced-Temperature of 33 °C, **g** 8 mM Gln at 37 °C, **h** 4 mM Gln at Reduced-pH of 6.8

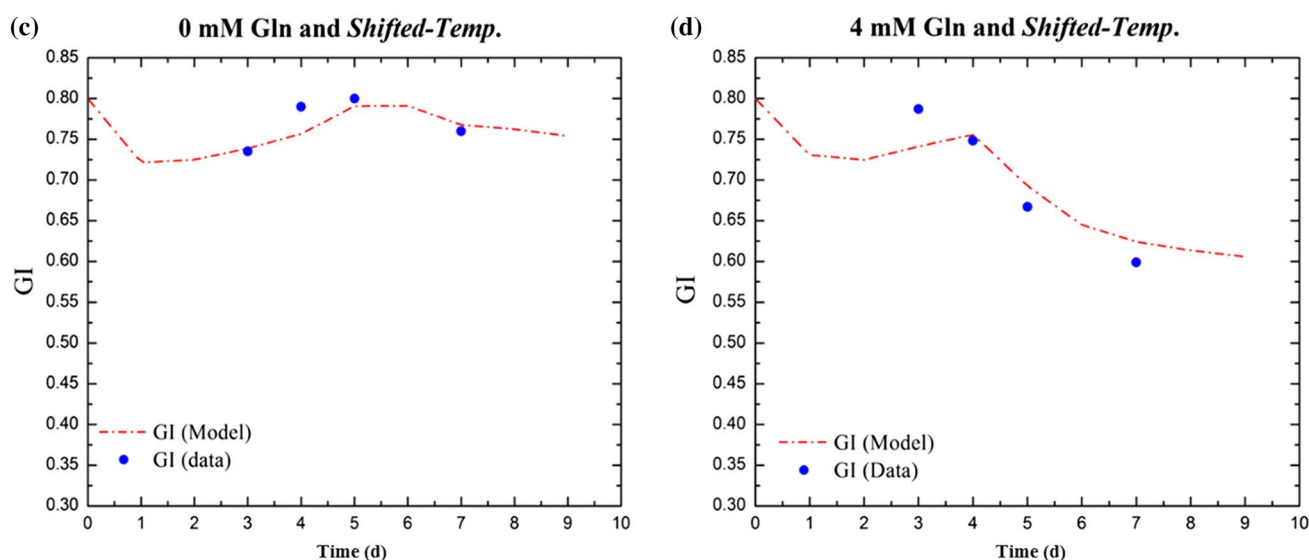


Fig. 8 Profile of experimental and predicted GI at different culture conditions for model prediction. Data points present experimental data and *dash lines* display simulated data as described in Table 1.

R^2 value calculated as 0.89. **c** 0 mM Gln and shifted-Temperature to 33 °C, **d** 4 mM Gln and Shifted-Temperature

4 mM Gln culture as compared to the 0 mM culture due to earlier depletion of Glc in the former (Fig. 7a, b). The largest errors between the model predictions and the GI measurements are observed for 0 M Gln and *Reduced-Temperature* and they are of the order of 5% of GI which is slightly larger than the experimental error in GI as identified in our previous work (~3%). Also, the specific mAb productivity is measured with ELISA that has an approximate measurement error of up to 15%. Another possible source of error is the initial value of GI that was assumed to be equal for all the cultures to the GI observed at day 3 for the Control culture, but it could not be directly measured due to the lack of sufficient amount of mAb at time = 0.

Conclusions

Cultivation of cells at mild hypothermia condition is a promising strategy for modifying mAb glycoforms during batch culture conditions. In these experiments, reducing temperature of the culture to 33 °C, either from the beginning (*Reduced-Temperature* strategy) or after the exponential phase of growth (*Shifted-Temperature* strategy) and regardless of the initial level of Gln, had a significant effect on cell growth, mAb productivity and glycosylation profile. Cell growth was, as expected, lower at 33 °C while cell viability remained high for a longer time of cell cultivation. The requirements of Gln and Glc for biomass formation seem to be different when operating at 33 °C versus normal temperature levels.

In this study, it was found that GI is significantly correlated to glucose and the specific growth rates but it is only slightly correlated to changes in mAb productivity. The proposed semi-empirical model was initially conceived to elucidate correlations between major culture parameters and the GI index. However, considering the simplicity of this semi-empirical model and its ability to describe the observed experimental trends, there is a potential to use it in the future for process optimization.

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