Quality-targeting Dynamic Optimization of Monoclonal Antibody Production

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Abstract: Compliance with Quality by Design (QbD) constitutes a major challenge in biopharmaceuticals. Monoclonal antibodies (mAbs) represent a significant biopharmaceutical product class, typically produced in mammalian cell cultures. A key quality attribute for mAb production is glycosylation. We examine how process intensification affects glycosylation via dynamic optimization using different problem formulations. We maximize process performance with simultaneous control of product quality. For these, we utilize a mechanistic dynamic model for mAb production in mammalian cell cultures including glycosylation presented by Ehsani et al. in *Computer Aided Chemical Engineering* (2017). To achieve target glycan distribution in the final product, we incorporate constraints for the acceptable glycosylation ranges into the dynamic optimization problem. As a result, we derive optimal supplementation profiles of nutrients and/or nucleotide sugars. This work successfully illustrates an example of how model-based dynamic optimization can be employed for implementation of the QbD approach in biopharmaceutics.

Keywords: QbD, monoclonal antibodies, dynamic optimization, glycosylation

Highlights:

- Shows an example of utilizing dynamic optimization for QbD in biopharmaceutics
- Examines problem formulations for high process performance considering product quality
- Derives optimal feeding profiles for mAb production with desired glycosylation ranges

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1 Introduction

Monoclonal antibodies (mAbs) constitute a rapidly-evolving high-value biopharmaceutical product with a wide range of applications. Therefore, many attempts have been made towards improving mAb process development and operation. In this direction, optimizing product yield has been on the main focus over a long period of time. As an outcome of those attempts, significant increase in product titers for mAb products has been accomplished [32, 41]. Current drivers in mAb processes point to the direction of flexible operation and shift of future process development from design of new technologies to comprehension and optimization of existing ones [32]. For this purpose, apart from achieving high product titers, efficiently monitoring and controlling of product quality comes into central focus [32, 41, 50].

To bring together regulatory authorities and pharmaceutical industry, the International Council for Harmonisation (ICH) was founded by the European Medicines Agency, the FDA and the Pharmaceuticals and Medical Devices Agency of Japan. ICH has established the Quality by Design (QbD) framework and the guidance documents that underly it, with the vision to develop more efficient processes that consistently meet quality specifications. However, in protein therapeutics the current methods in this direction are still in an early stage [45]. While process development is still quite empirical, advancements in process analytical technologies and chemometric methods have facilitated a deeper process understanding, have significantly reduced the analysis time and have by now been successfully established as common practice in biopharmaceutical manufacturing [50, 11, 45]. To this end, knowledge on qualitative structure characteristics is an additional tool for risk assessment and improving early process development [30].

The application of the QbD framework necessitates identification of the critical quality attributes (CQAs) and of suitable variables for manipulating the CQAs to their desired states [13, 45]. Glycosylation, aggregation and charge isoforms are reported in the literature as CQAs in mAb production [31]. The work in [31] shows that process conditions do impact product quality. Sharfstein et al. (2008) [50] review literature attempts on understanding the impact of culture conditions on productivity and product quality. Although basic understanding and some first links between CQAs and appropriate variables for their control in protein therapeutics are being established, moving from process knowledge to CQA controllability remains still a major challenge [7, 45].

In protein therapeutics, one of the most critical and sensitive quality attributes is glycosylation [7, 8, 41, 45, 55], which is a complex post-translational modification that takes place in the endoplasmic reticulum (ER) and Golgi apparatus of a cell [7]. It refers to the addition of glycan structures to polypeptide chains and can influence physico-chemical properties and the clinical action of protein therapeutics, namely safety and efficacy of the drug [7, 45]. Particularly in the case of mAbs, it directly affects the immune effector function of therapeutics [8]. Consequently, glycosylation characteristics derive from specific knowledge about desired phenotypes, and acceptable limits on glycosylation variability are established during preclinical and clinical assessment of therapeutic proteins [7]. To ensure consistency of product quality during commercial manufacturing, these limits should be satisfied.

Glycosylation profiles can be influenced by a series of factors, among which

cell line, culture conditions, mode of operation and down-stream processing [9, 45]. During late stage process development and optimization, the cell line is already selected, and the variations in the purification process only affects the relative proportions of the glycan structures. Therefore, at these stages, culture conditions that significantly affect glycan biosynthesis are of more immediate interest. The effects of culture conditions on glycosylation, as well as of glycan heterogeneity on various antibody effector functions have been the focus of many research studies. Batra et al. (2016) [7] review current developments and outcomes on this field. Research findings indicate the existence of a trade off between cell growth, mAb productivity and increased glycosylation levels [3]. Deriving appropriate feeding of nutrients and nucleotide sugars (NSs) is a promising way to achieve the desired balance between these goals. Mathematical models can be exploited in order to identify optimal feeding profiles, fill the existing gaps in process knowledge, and avoid time- and labor- intensive analysis [45].

Monoclonal antibody production is typically carried out in mammalian cell cultures, due to their ability to effectively perform post translational modifications, such as glycosylation [6, 50, 51]. Over the years, different cell culture models with different levels of complexity and biological insight have been created. Although these models are primarily built to predict process behavior, recently such models have been also successfully used for computationally intense optimization studies, e.g., [12, 14, 29, 35]. However, these models so far did not include direct measures for product quality, and could therefore not be used for quality-targeting optimizations.

To address the QbD challenge in protein therapeutics, mathematical models that describe the glycosylation process have also been developed, e.g., [17, 37]. This paves the way towards model-based optimization that can directly account for product quality attributes. In the past years, some first studies towards understanding the effects of process changes in glycosylation within the paradigm of QbD for protein therapeutics are undertaken. Green and Glassey (2015) [22] perform a multivariate analysis that looks at modeling of the effect of operating conditions on glycosylation. Aghamohseni et al. (2014) [3] utilize mathematical modeling and experimentation to quantify the impact of nutrient levels on glycosylation and mAb productivity. Villiger et al. (2016) [55] employ a high-throughput method for screening nucleotides and nucleotide sugars to explore their influence on glycosylation. Recently, Ehsani et al. (2019) [16] utilize design of experiments and model simulations to investigate the effects of feeding scenarios on the protein glycosylation pattern using a calibrated model. Kotidis et al. (2019) [38] develop a predictive mathematical model for cell culture dynamics and mAb glycosylation. This model was used to define an optimal uridine/manganese/galactose feeding strategy for optimization of antibody galactosylation, and the optimization results are then validated with independent experimentation.

Two of the most typical operation modes for mAb production are fed-batch and perfusion systems [41]. Although increased research emphasis is currently placed on continuous manufacturing [31, 36], fed-batch operation still reflects the common choice for large scale production, mainly due to certain advantages it offers, such as scalability, operational simplicity and high productivity [41].

In this work, we perform model-based optimization to devise quality-consistent feeding strategies for intensification of mAb producing processes in fed-batch

mode of operation. In a first step, we examine effects of nutrients supplementation on antibody glycosylation, when using dynamic optimization to increase the final product. The results, as presented in Section 4, confirm the variability of glycosylation when changing from batch to fed-batch mode. Therefore, in a next step, dynamic optimization of mAb production with simultaneous consideration of glycosylation-associated product quality is employed. More precisely, we derive optimal feeding profiles for nutrients and nucleotide sugars, accounting at the same time for both increased production outcome and glycoprotein distribution within specified acceptable ranges reported in the literature. To the best of the authors' knowledge, it is the first time that multiple glycosylation related quality attributes have been incorporated as constraints to the dynamic optimization problem to successfully maximize antibody production while satisfying product quality.

The rest of the paper is structured as follows. Section 2 illustrates the basic characteristics of the model utilized for the optimization studies, as well as the procedure for parameter identification and parameter fitting to experimental data from the literature. In Section 3, we describe the different formulations to the dynamic optimization problems, first for monitoring (Section 3.1) and later for controlling (Section 3.2) product quality, in terms of target glycan distribution. We present and discuss the results of these optimizations in Section 4, and in Section 5 we conclude this work.

2 Modeling and parameter identification

To efficiently model and control product quality, we need to describe and connect the different scales of the cell culture process. To this end, our mechanistic dynamic model for the production of mAbs in mammalian cell cultures presented in Ehsani et al. (2017) [17] serves as basis for this work. The model is comprised of three major layers including bioprocess dynamics, intracellular reaction network and kinetic reactions inside Golgi apparatus. The model consists of 271 differential equations, 374 algebraic equations and 155 parameters. A brief description of the model equations presented in [17] is provided in Appendix A.

In this study, the presented model is used to investigate the effect of nutrient supplementation in the process and product performance. Along with nutrient availability, other bioprocess conditions such as pH, temperature and dissolved oxygen affect the cell culture, and thus the glycosylation process [13]. These effects are not incorporated to the model.

In order to identify the most sensitive model parameters, we first perform global sensitivity analysis (GSA) using random sampling - high dimensional model representation (RS-HDMR) [56] by evaluating 10^5 samples in the range of 50 % - 200 % of the parameters' nominal values. Note that the reported nominal values do not lie not on the arithmetic mean of the distribution but rather the geometric one. Due to the presence of stiff ordinary differential equations in the examined model, simulation with parameter values below the imposed limit of 50 % of the nominal values results to either abnormal termination or very long CPU times. Hence, to avoid simulation problems with parameter values in the range of 0 % - 50 % of the nominal values, the analysis is carried out in the reported range. To expand RS-HDMR function, we use third-order Legendre

polynomials for the uniformly distributed samples in the parameter space.

In a next step, we utilize a scatter search algorithm [15] to tune the sensitive parameters identified by GSA. For the unstructured part (first two layers) experimental data taken from [26] are used for the tuning. The experimental data taken from [26] include some key bioprocess indicators and metabolites in an immunoglobulin (IgG1) producing mammalian cell culture (e.g., viable/dead cell density, glucose, glutamine, lactate, ammonia, mAb), which are used to tune the first model layer, as well as the concentrations of nucleotide sugars, which are integrated to tune the second layer parameters. The experiments are conducted in a batch culture, which defines a unique dataset for our study, and provides a good starting point to investigate the effect of nutrient supplementation in the culture. More precisely, 20 parameters (these refer to the reduced number of parameters after the GSA) are estimated using the data reported from analyzing 12 metabolites in 13 time points. For the third layer, namely the kinetic reactions inside Golgi apparatus, we use a different time course dataset of glycosylation profiles for the fitting, derived from [25]. Both above-mentioned data sources deal with the same isotype of mAbs, i.e., IgG1. IgG1-based mAbs constitute the majority (> 70 %) of clinically approved mAbs [13]. Furthermore, glycosylation profiles have in general large overlaps for different mAb products, and thus their major glycoforms fall often into the same specific ranges, e.g., [7]. These observations enable us to combine the two different datasets for model fitting. Of course, when available, data from the same source is preferred. However, due to the lack of reported time course data for the glycoform variations during a mAb production cell culture process published in the literature, this was not possible in our case. In the third layer of the model, 25 parameters are estimated using the data reported in [25]. Here also an IgG1 producing mammalian cell culture (without supplementing metabolites for optimization of glycan profile) is used to estimate the parameters, and to simulate the system as a starting point for evaluating the effects of supplementing sugars and nucleotide sugars. This dataset includes the measurements of 13 glycan structures in 13 time points.

3 Process Optimization

Optimal feeding profiles that provide cell culture with the appropriate amounts of nutrients can essentially contribute to an increased production outcome. However, overfeeding can have an inhibitory effect on cell growth, mainly due to the accumulation of toxic byproducts. Furthermore, supplementation affects quality attributes such as glycosylation. More precisely, nutrient feeding leads to changes in nucleotide sugars metabolism, and thus to the glycosylation patterns. Thus, the dynamic nature of the described problem necessitates dynamic optimization techniques, in order to derive optimal feeding strategies that achieve high product formation and simultaneously comply with the required quality attributes.

In our model, as originally presented in [17], the unstructured cell culture part describes viable cell density, extracellular metabolite and nucleotide sugars concentrations, as well as specific productivity. Specific productivity and nucleotide sugars concentrations are the input parameters to the glycosylation part. Assuming each protein carries two glycan molecules (one M9 and one M8)

on its Fc region, the production rate of each of these two glycan is equal to the specific protein production rate. Since the protein secretion process occurs after glycosylation in Golgi apparatus [53], the rate of protein production influences protein glycosylation, and the glycosylation profile affects the rate of recombinant protein secretion as a quality criterion. The mechanisms that underlie the impact of specific productivity on glycosylation have been extensively discussed (using a model-based approach) in [27]. To this end, process optimization is crucial in order to derive trade off between mAb productivity and high glycosylation processing levels. Noticeably, the results in [22] indicate the potential of predicting most glycan forms at the end of the cultivation through glucose (Glc) and amino acids concentrations as well as product titre.

Some indicative values on acceptable ranges for glycoprotein distribution of mAbs are reported in the literature [7]. Starting with a batch process that gives glycan distribution very close to these ranges (unoptimized batch case), we perform model-based dynamic optimizations. We firstly examine the effects of process optimization on the distribution of glycoforms, by supplementing nutrients. To ensure variability of glycoprotein distribution within acceptable ranges, we then reformulate the optimization problem to control glycosylation ranges. For this, both feeding of nutrients and nucleotide sugars is employed.

The categorization of the glycan structures to quality attributes can be seen in Figure 1.

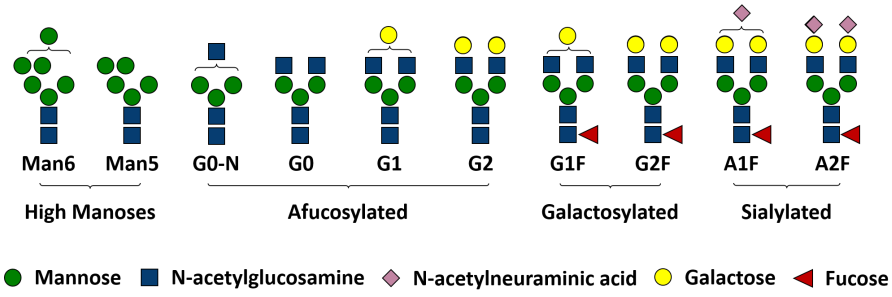

Fig. 1:
Categorization of glycan structures to quality attributes.

In Table 1 we present a brief summary of the examined case studies. Case studies CS1-3a and CS1-3b refer to optimizations without and with quality attributes, respectively.

We solve the resulting dynamic optimization problems using direct single-shooting method. A piecewise constant discretization of the controls is employed, where the time horizon is discretized to six-hour intervals, with enabled grid adaptation. The optimizations are performed within gPROMS ModelBuilder v.5.0.1 [1]. Optimization tolerances are set to 1E-3. More information about CPU times and optimization status is presented in Table B1 in Appendix B.

3.1 Process optimization without control on glycosylation

Starting from an unoptimized batch case, we explore different scenarios for the optimization of the fed-batch case. In all optimizations we consider supplemen-

Table 1: Summary of examined case studies

	Objective	**Controls**		
	Maximize	Feeding Glc, Gln	Feeding Nucleotide Sugars	Initial Concentrations Glc, Gln
CS1a	final mAb concentration	Yes	No	No
CS1b	final mAb concentration	Yes	Yes	No
CS2a	final mAb concentration	Yes	No	Yes
CS2b	final mAb concentration	Yes	Yes	Yes
CS3a	volumetric productivity	Yes	No	Yes
CS3b	volumetric productivity	Yes	Yes	Yes

tation of glucose and glutamine (Gln), which correspond to the main carbon and nitrogen sources for the cells, respectively. In our first case study (CS1a) we consider, as commonly done [29, 35, 40], only feeding rates and composition of Glc and Gln as optimization variables. We aim at maximizing the mAb final product concentration, which is our desired high-value biopharmaceutical product. The second case study (CS2a) has the same objective as in CS1a, but considers additionally initial concentrations of Glc and Gln in the culture as optimization variables, since medium composition is crucial for cell growth and product formation. The benefits of using alternative objectives have been also reported by us and others [28, 39]. Thus, in the third case study (CS3a), we present optimizations using volumetric productivity as an objective, varying again initial nutrient concentration and feeding of Glc and Gln.

The upper bound on the inlet flow rates derives from the imposed constraint on maximum allowed feeding volume as presented below (feeding rate cannot be greater than the considered volume variation throughout the culture). The upper bounds of Glc and Gln concentrations in the feed are selected ad-hoc based on values reported in the literature [35, 47]. The culture time is an additional degree of freedom and is flexible to vary from one to twice the batch time, as long as all imposed constraints are satisfied. Our previous experience has shown that this bound is sufficient, as process optimization typically cannot extend culture longevity that much [28, 29].

We formulate, as state constraint, an upper bound on the integral of the feeding rate (boundary value for maximum volume variation from [39]). We also impose state constraints on the upper bound of lactate and ammonia concentration, which are toxic byproducts (bounds reported in [58]), and on the lower bound of culture viability (value from [48]) to avoid undesired product degradation. Viability is the percentage of viable over total cells in the culture, and its lower bound usually serves as termination criterion for the optimization. In many practical applications a higher bound on viability throughout the culture is maintained, cf., e.g., [31]. As our study focuses on the early steps of

process development within the QbD framework, we believe it is important to explore the process behavior in the regions of low viability index to gain valuable process understanding and enable the assessment of potential issues in future steps of process development. Note that a linear correlation with increasing the lower bound on viability and decreasing both final product titer and culture duration is reported in [29]. Finally, we introduce an upper bound to the Glc concentration level throughout the cultivation, at the value of the initial Glc concentration in the examined batch case. This is because presence of Glc in abundance has been reported as energy inefficient [34].

A mathematical formulation of the problem description above is given by the following set of equations (1):

$$\begin{aligned} \max_{u(t)} \quad & ProcessObjective \\ s.t. \quad & CultureViability(x(t), u(t)) \geqslant 50\% \\ & VolumeVariation(x(t), u(t)) \leqslant 20\% \\ & Amm(x(t), u(t)) \leqslant 5.1mM \\ & Lac(x(t), u(t)) \leqslant 58mM \\ & Glc(x(t), u(t)) \leqslant Glc_0 \end{aligned} \tag{1}$$

where $x(t)$ state variables, $u(t)$ the degrees of freedom: F_{inGlc}, F_{inGln} [0-0.08] (L/h), c_{inGlc} [0-400] (mM), c_{inGln} [0-200] (mM), t_f [94.8-189.6] (h) and $mAb(t_f)$ the final product concentration. For CS2 and CS3 we have additional c_{0Glc} [0-24.2] (mM), c_{0Gln} [0-10] (mM).

As presented in the results section, although these optimizations improve the process objective, the final product does not meet the target glycan distribution. This raises a critical point for practical implementation, as it implies that optimization studies relying solely on an increased production outcome through nutrient supplementation without directly accounting for product quality, can fail to deliver a high quality glycosylated product.

3.2 Process optimization with control on glycosylation

To be able to fulfill target glycan distribution for the final product, we develop case studies (CS1-3b), where we now incorporate directly product quality specifications (in terms of acceptable glycosylation ranges) to the previously examined optimization problems.

The following reformulations of the optimization problem are performed.

Glycosylation ranges at the end of the culture are included as constraints. This is reasonable, as we examine batch and fed-batch cultures, where the product is only collected at the end of the culture life span. In practice, mAb secretion occurs throughout the culture time, and thus in the future additional "control points" could be incorporated. However, this would require some additional information on the propagation of acceptable glycosylation ranges with respect to the dynamics of the glycosylation process throughout the culture, which to the authors' best knowledge is not yet available in the literature.

Additional feeding of nucleotide sugars is introduced to manipulate glycoprotein distribution to the desired ranges. More precisely, we consider feeding of UDP-GlcNAc, UDP-Gal, GDP-Fuc, since the significance of the variations in

glycan profile, aroused from changes in the concentrations of these nucleotide sugars are the most frequently reported in the literature, cf., e.g, [8, 55].

Feeding galactose, N-Acetylglucosamine and fucose are reported to dramatically increase the intracellular concentrations of UDP-Hex (UDP-Glc and UDP-Gal), UDP-GlcNAc and GDP-Fuc, respectively [5, 18, 52, 55, 57]. Hence, in our model simulations we assume that feeding these supplements indirectly reflects the effect of supplementing the respective sugars. This assumption enables us to compare our results against the ones reported in the literature. However, moving from extracellular metabolites to intracellular nucleotide sugars actually corresponds to complicated metabolic pathways that depend on numerous mechanisms and process conditions. Thus, a more accurate calculation of nucleotide sugar bounds could in a future step derive based on maximum uptakes of nutrients and stoichiometry of metabolic reactions.

Alongside feeding the carbohydrate component of nucleotide sugar donors (NSDs), it is also essential to feed nucleotide precursors, such as uridine and guanosine (for UDP-GlcNAc, UDP-Gal and GDP-Fuc, respectively). Feeding of these nucleotides is known to inhibit cell growth [10, 21]. Model-based optimization of NSD feeding strategies has been recently published [38]. In the present study, feeding of these factors is not considered, assuming they are provided in the media or supplemented feed. Hence, the potential deleterious effects of nucleotide precursor feeding have been neglected. In future studies such effects should be also considered.

The upper bounds on the feeding rates derive again from the constraint on maximum culture volume and the feeding concentrations of the nucleotide sugars from the values reported in [55]. Note that in CS2 an additional constraint to avoid feeding when viability level has dropped bellow 70 % is imposed. In the following section, the incorporation of this constraint is justified.

4 Results and discussion

In this section, we present the optimization results for the examined case studies. In the first subsection, we observe general trends and discuss the effects of utilizing different problem formulations on optimization outcomes. In the second subsection, we focus more on the quality aspects, evaluating the impact of process optimization to the considered quality criteria for the different case studies.

The key indicators of the optimization results are summarized in Table 2. Figures 2,3,4 illustrate the time evolution of some key process variables for CS1, CS2 and CS3, respectively. The optimal feeding profiles and overall volume variation for the optimizations without and with quality attributes for the examined cases, the glycan distribution profiles, as well as the optimal feeding concentrations are presented in the Appendix B (Figures B.1,B.2,B.3, Figures B.4,B.5,B.6 and Table B2, respectively).

The results illustrated here represent the solutions to a mathematical problem, as this is described in the previous section. In the following, the obtained results are discussed in terms of process.

Table 2: Optimization results. HM is high mannoses, aFuc is afucosylated, Gal is galactosylated, Sial is sialylated. Values with * indicate an active bound. Values with ** indicate that the specification is out of range.

	HM %	**aFuc %**	**Gal %**	**Sial %**	**Final mAb [mg/L]**	**Culture Time [h]**
Acceptable Range([7])	3-10	2-13	10-40	0-2	N/A	N/A
Unoptimized Batch	11.2**	2.5	28.3	0.3	124.3	94.8*
CS1a	8.0	0.0**	28.6	0.3	137.8	94.8*
CS1b	4.7	2.0*	29.2	0.3	138.1	94.8*
CS2a	57.1**	0.1**	16.4	0.4	138.1	104.6
CS2b	10.0*	2.0*	10.0*	0.1	141.3	189.6*
CS3a	2.5**	0.0**	6.3**	0.1	137.4	94.8*
CS3b	3.0*	2.0*	18.1	0.2	137.4	94.8*

4.1 Comparison of the optimization results for the different case studies

From the results presented in Table 2, we observe that in all cases although optimization without direct quality attributes (optimized without QAs) leads to an increase of product titer, the glycan distribution of the optimized cases does no longer lie within the acceptable ranges. This is expected, since as already discussed, operation mode directly affects glycosylation. As already mentioned, this practically means that optimization studies considering only maximization of a process objective would fail to deliver a quality compliant product. When optimization with constraints on glycosylation ranges is conducted (optimized with QAs), all glycosylation ranges are respected, and still the final product concentration is at the same levels as the one from optimization without quality attributes (see Figures 2a,3a,4a). This is a significant finding, as it indicates that we do not necessarily need to sacrifice process performance to achieve high quality. This of course requires careful selection of the manipulated variables and their imposed bounds, as well as further experimental studies to support this claim.

At this point it is to be noted that transforming the optimization problem into a multi-objective formulation poses an interesting alternative, to the approach considered here, namely including glycosylation ranges as endpoint constraints. This might be of a specific interest once a desired clinical effect of the mAb is identified, e.g., maximize galactosylation content to increase complement dependent cytotoxicity (CDC) activity [13]. Such cases are further discussed in the next subsection. Yet, from a mathematical perspective, formulating the

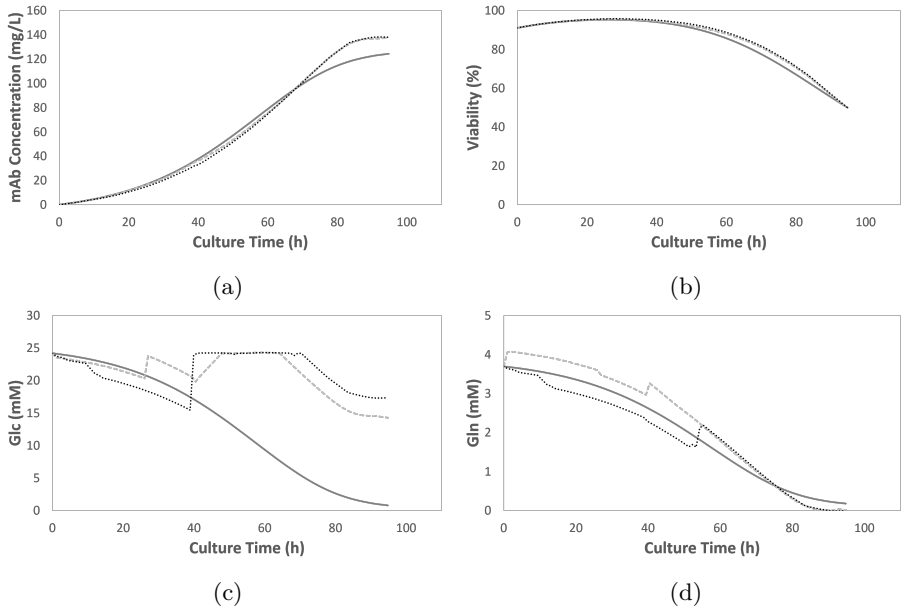

Fig. 2:
CS1 - Comparison of key indicators for unoptimized (solid, dark grey) optimized without QAs (dashed, light grey) and optimized with QAs (dotted, black) cases.

quality attributes as constraints with desired ranges offers a considerable advantage, as it gives additional degrees of freedom to the optimization problem. This practically means that the optimization strategy can decide where on those ranges maximum process performance is attained.

Comparing CS1 and CS2, we notice that the additional degrees of freedom (initial concentrations of Glc and Gln) in CS2, lead to significant violation on the final glycosylation profile of the product (see Table 2) for the optimizations without QAs. For our *in silico* studies, the optimized results of CS2b play a significant role as, despite the large deviations from the desired glycopatterns presented in CS2a, they manage to meet all imposed glycosylation specifications for the final product, while also increasing the final product titer. However, these results do not look favorable for practical implementation, due to the following reasons. First, by incorporating feed towards the end of the culture, the cells most probably won't be able to recover (results not presented here). To avoid this behavior, a constraint to eliminate feeding at a low viability levels is added (see Figure 3b). Yet, Gln concentration still remains zero for a prolonged period of time (see Figure 3d), which since Gln is crucial for the survival of the cell culture is not desired. Moreover, the optimized feeding strategy considerably prolongs culture time without leading to significant increase of the product concentration. In order to remedy this, we examine in CS3 an objective that takes into account culture time, namely volumetric productivity. In certain cases, maximizing specific productivity might also be of interest. However, in our case this leads to low cell densities in the optimization, and thus low titers (results not presented here).

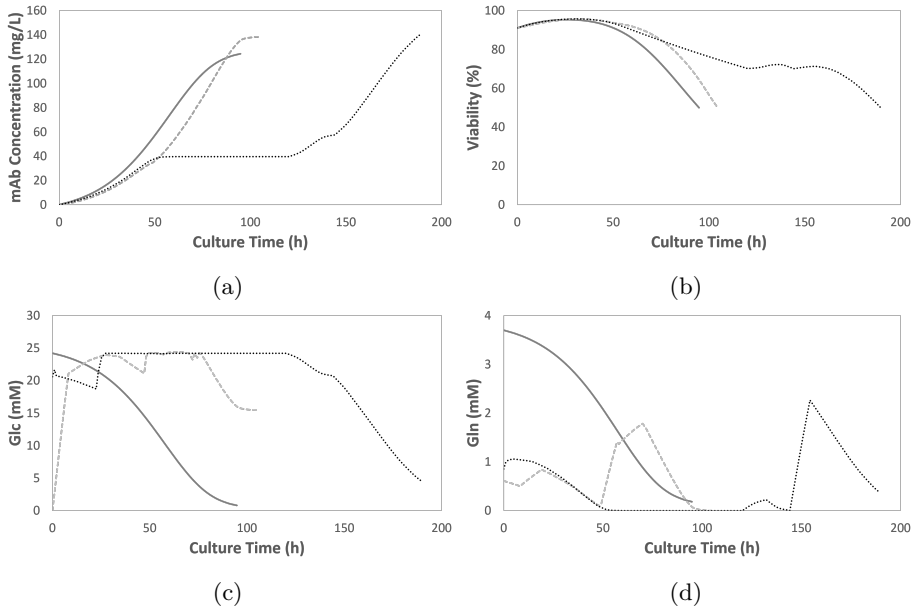

Fig. 3:
CS2 - Comparison of key indicators for unoptimized (solid, dark grey) optimized without QAs (dashed, light grey) and optimized with QAs (dotted, black) cases.

Optimization with CS3 shows an example of how alternative objectives might be used for increased production outcome. Consideration of production time within the objective function leads to culture longevity at the lower bound (in this case batch time). However, as the optimized final product concentration of CS3 is still comparable with the optimized one from utilizing an objective specifically focusing on final product concentration (CS1, CS2), consideration of volumetric productivity as an objective could be also considered in practice. Note that in general for case studies CS1a, CS1b and CS2a a prolonged culture duration when moving from batch to fed-batch operation is anticipated. The deficiency of our optimization results to capture this behavior is to a great extend attributed to the limitations of the obtained parameter set to efficiently meet all system requirements, taking also into account the imposed lower bound on viability and the data described in [26].

Among the presented case studies, CS1b is deemed as the most promising one, since it combines an increased product titer with desired glycosylation patterns, namely high galactosylation index and low fractions of high mannoses and afucosylated glycan structures. Please note that the potential influence of the selected objectives to process scale up is considered out of the scope of the presented study, and thus not presented here.

4.2 Effects of optimizations on glycosylation

The biantennary glycans G0F (G0 refers to the number of galactose on the two antennas and F refers to the fucosylation of the first N-acetylglucosamine, as

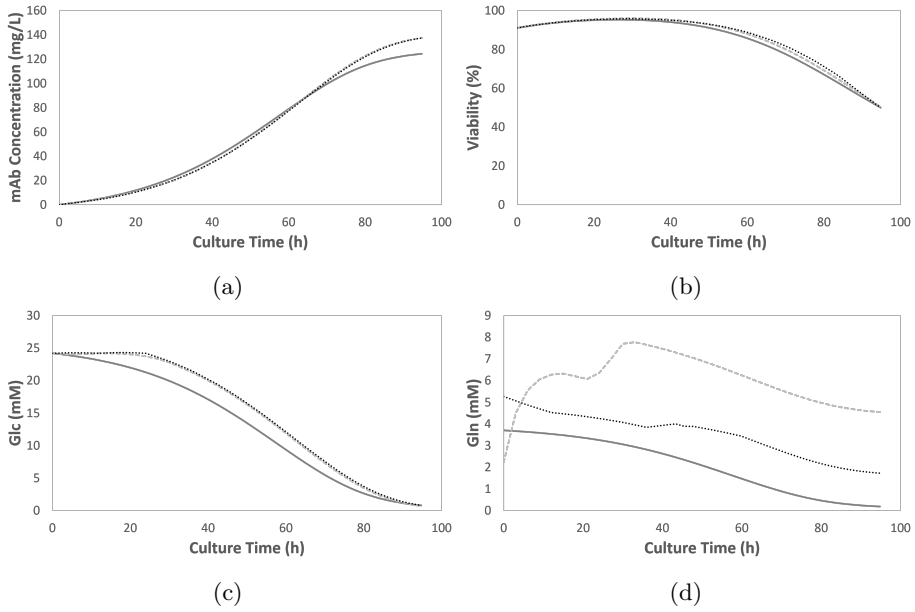

Fig. 4:
CS3 - Comparison of key indicators for unoptimized (solid, dark grey) optimized without QAs (dashed, light grey) and optimized with QAs (dotted, black) cases.

represented in Figure 1) and the galactosylated structures G1F-G2F are critical in glycosylation, and they represent a major fraction of the total glycosylation profile of therapeutic antibodies [23, 49]. Supplementation of glucose and galactose is expected to increase the fraction of G0F and G1F-G2F [33, 43]. However, galactosylation content stays generally in our optimization at relative low levels (yet apart from CS3a still within the acceptable ranges), which could be a critical point as in most cases higher galactosylation levels are more favorable [8, 19] (see also Figures B.4c,B.5c,B.6c). To avoid this behavior we could either increase the lower bound on the acceptable galactosylation range (using as reference the galactosylation levels of popular mAb products, the glycan profiles of which are available online e.g., [19]) or incorporate galactosylation content into the objective function (multi-objective formulation). A possible reason for the decreased galactosylation content in our optimization could be high amounts of GlcNAc. The increase in GlcNAc availability due to supplementation seems to induce higher formation of G0F and the galactosylation content remains at low levels. Although the inhibitory effects from transport of nucleotide sugars are not considered in our mechanistic model, it is reported in the literature that the increase in GlcNAc transport into the Golgi (when available in high concentrations) slows down the transport of UDP-Gal [55, 57].

It has been (frequently) reported that the presence of high mannose structures over a specific range increases the antibody clearance from human body [4, 20, 42]. Moreover, the human immunoglobulin G (IgG) contains very low content of high mannose glycans compared to recombinant monoclonal antibodies glycan profile [20]. The presence of elevated high mannose glycans is

typically an indication for early departure from glycosylation [8]. For all the above reasons, minimizing the high mannoses content is desired in mAb glycosylation process. In our optimization, by supplementing more carbon sources (Glc and Gln), we observe a generally decreasing trend in the high mannoses content, compared to the unoptimized batch case (except CS2a, where there is a channeling through high mannoses production, indicating that the network does not progress to mature glycans). In all cases where we included the adjustment of the glycosylation profile to the desired ranges, the high mannoses content is significantly decreased. Figures B.4a,B.5a,B.6a illustrate the variations in the high mannoses content for the different case studies. From these results, we can observe that even in the optimized case studies, where an upper bound to reduce the high mannose glycans in the final product is imposed, high mannose species accumulate to up to 30 % during the initial phase of culture (e.g., Figure B.4a). This profile deviates from what was previously reported in [54], where the high mannose glycan profiles begin at ca. 5 % and subsequently increase to 25 % during the second half of culture. As the dataset used for the tuning of the third model layer only reports data from the third day on, the dynamics of the glycosylation process at the early culture stages in our in silico study are expected to have higher deviations from reality. Since also we only impose the constraints to the glycosylation ranges at the final point, the profiles of the optimized cases do not reduce the high mannose content at early stages. However, in the late culture times, where also our constraints are imposed, the results are expected to be more reliable.

As already mentioned, the major glycan contents in therapeutic mAbs are the core fucosylated structures G0F and G1F-G2F. However, for some antibodies (those whose mechanism of action includes antibody dependent cell mediated cytotoxicity) the lower fucosylation content enhances the mAb activity and efficacy strongly [2, 44]. Therefore, maintaining the afucosylated content within the desired ranges is of high importance. Our optimization indicates a trend towards minimizing afucosylation content, yet keeping it within the imposed bounds when quality constraints are introduced (Figures B.4b,B.5b,B.6b).

Although the relative percentage of sialylated glycans in monoclonal antibodies is low [52] the progression of glycosylation process to the increased level of antennarity and capping with sialic acid prevents fast removal of protein from blood circulation and therefore increases serum half-life [46]. It is therefore crucial to keep the sialylation levels within the desired ranges. In all our optimization cases, the target sialylation context is achieved (also for the optimizations without incorporation of the constraints on the glycosylation ranges) (see also Figures B.4d,B.5d,B.6d). This is most probably due to the imposed ammonia constraint, which maintains low ammonia concentration in the culture, since increased ammonia levels have been reported in the literature to have negative effects on sialylation and galactosylation [3, 7].

5 Conclusion

We dynamically optimize feeding profiles of nucleotide sugars and/or essential nutrients in order to increase mAb titer in mammalian cell cultures, taking into account product quality. Quality attributes, in our case studies, refer to target glycan distribution of the final product. Switching from batch to fed-batch

operation, cell glycosylation is affected, and thus quality-based optimization is required to be able to maintain acceptable glycosylation ranges for mAb production. This is done by including them as constraints to the optimization problem formulation.

The results of our in silico study confirm an increased production outcome, which satisfies the imposed quality constraints. Although no experimental validation of the results is provided, this work shows an effective methodology, with which quality attributes can be incorporated into the optimization problem increasing final product titer, while simultaneously meeting target glycoprotein distribution. It additionally highlights the multiple benefits of utilizing mathematical models for process optimization in biopharmaceutics, within the broader concept of QbD paradigm. Yet, as the validity of model-based optimization strategies is to a great extent correlated to the model accuracy and efficacy, further attempts towards the development of predictive mathematical models that can effectively capture process behavior are needed. This can minimize experimental efforts and provide a valuable process insight. In this direction, data generation, availability and methods for its successful utilization is a vital element.

In the future, more research emphasis should be placed on investigating the effect of other growth-limiting and/or growth-facilitating substances and supplementing them into the culture. This can facilitate process intensification (as more biomass can lead to more product), and also provide more efficient mechanisms for controlling the glycosylation process. Additionally, other quality-related attributes can be incorporated. Last but not least, experimental validation of the results is required.

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A Model description

In the following, a short description of the model equations given in Ehsani et al. (2017) [17] is provided.

Cell culture model

The dynamics of the cell culture are described as functions of extra- and intracellular metabolites. The major nutrients (i.e., Glc and Gln), inhibitory substances (i.e. Lac and Amm) and the glycoprotein constitute the extracellular metabolites. The nucleotide sugars are the intracellular metabolites considered in the model. The mass balances are written assuming perfect mixing.
The volume (V) varies based on the feed rates (F_{in}) and the outlet flow (F_{out})

$$\frac{dV}{dt} = F_{in} - F_{out} \tag{A.1}$$

The balances on the viable (X_v) and dead cells (X_d) are functions of the specific growth (μ) and death rates (μ_d). The lysis rate is also considered in the dead cells balance

$$\frac{d\left(V \cdot X_v\right)}{dt} = V \cdot X_v \cdot (\mu - \mu_d) - X_v \cdot F_{out} \tag{A.2}$$

$$\frac{d\left(V \cdot X_d\right)}{dt} = V \cdot X_d \cdot (\mu_d - k_{lys}) - X_v \cdot F_{out} \tag{A.3}$$

The growth rate is described as a Monod function of Glc, Gln, their saturation coefficients and the maximum growth rate. The growth inhibiting effects of Amm and Lac are also considered by Monod kinetics

$$\mu = \mu_{max} \cdot \frac{[Glc]}{k_{Glc} + [Glc]} \cdot \frac{[Gln]}{k_{Gln} + [Gln]} \cdot \frac{k_{i,Lac}}{k_{i,Lac} + [Lac]} \cdot \frac{k_{i,Amm}}{k_{i,Amm} + [Amm]} \tag{A.4}$$

The death rate is a function of Amm concentration in the culture, the maximum death rate and the Amm constant for cell death

$$\mu_d = \mu_{dmax} \cdot \frac{1}{1 + \left(\frac{k_{d,Amm}}{[Amm]}\right)^2} \tag{A.5}$$

The mass balances of Glc and Gln into the bioreactor are given by

$$\frac{d\left(V \cdot [Glc]\right)}{dt} = -V \cdot X_v \cdot Q_{Glc} + F_{in,Glc} \cdot [Glc_{in}] - F_{out} \cdot [Glc] \tag{A.6}$$

$$\frac{d\left(V \cdot [Gln]\right)}{dt} = -V \cdot X_v \cdot (Q_{Gln} + Q_{d,Gln}) + F_{in,Gln} \cdot [Gln_{in}] - F_{out} \cdot [Gln] \tag{A.7}$$

The specific consumption rates of Glc and Gln are functions of growth rate, their biomass yield coefficient and their maintenance term

$$Q_{Glc} = \frac{\mu}{Y_{X_v/Glc}} + m_{Glc} \tag{A.8}$$

$$Q_{Gln} = \frac{\mu}{Y_{X_v/Gln}} + m_{Gln} \tag{A.9}$$

The rate of degradation of Gln in the culture media is described by a first order kinetic rate

$$Q_{d,Gln} = k_{d,Gln} \cdot [Gln] \tag{A.10}$$

The mass balances for lactate and Amm are given by

$$\frac{d\,(V \cdot [Lac])}{dt} = V \cdot X_v \cdot Q_{Lac} - F_{out} \cdot [Lac] \tag{A.11}$$

$$\frac{d\,(V \cdot [Amm])}{dt} = V \cdot X_v \cdot Q_{Amm} + k_{d,Gln} \cdot V \cdot [Gln] - F_{out} \cdot [Amm] \tag{A.12}$$

where

$$Q_{Lac} = Y_{Lac/Glc} \cdot Q_{Glc} \tag{A.13}$$

$$Q_{Amm} = Y_{Amm/Gln} \cdot Q_{Gln} \tag{A.14}$$

The glycoprotein balance is described as follows

$$\frac{d\,(V \cdot [mAb])}{dt} = V \cdot X_v \cdot Q_{mAb} - F_{out} \cdot [mAb] \tag{A.15}$$

where the specific production rate is given by

$$Q_{mAb} = \frac{\mu}{Y_{X_v/mAb}} \tag{A.16}$$

The four considered nucleotide sugars, namely UDP-GlcNAc, UDP-Gal, GDP-Fuc and CMPNeu5Ac are modeled with the following general mass balance equation

$$\frac{d\,[NS_i]}{dt} = \sum_{j=1}^{\#reactions} S \cdot r_{i,j} - M_{cell,i} - Trans_{Golgi,i} \tag{A.17}$$

where the intracellular biosynthetic rate ($r_{i,j}$) and transport reactions into Golgi ($Trans_{Golgi,i}$) are presented by Monod kinetics, S is the matrix of stoichiometric coefficients, and $M_{cell,i}$ is the maintenance term, which represents the consumption of nucleotide sugars in the host cell proteins glycosylation for each nucleotide sugar.

Protein glycosylation model

Assuming an efficient and complete glycosylation process in ER, the protein carrying glycan molecules enters the Golgi apparatus. The rate of glycan structures entering Golgi is derived from the specific production rate (Q_{mAb})

$$[G_{1,1}] = 2 \cdot \left(\frac{Q'_{mAb}}{MW_{mAb}}\right) \cdot \left(\frac{V_{cell}}{V_{Golgi}}\right) \tag{A.18}$$

where (Q'_{mAb}) is the specific production rate in μM. Considering that each protein contains two N-linked oligosaccharides, one on each of the two heavy chains (on the $C\gamma 2$ domain) that compose the full mAb molecule, the whole term is multiplied by 2. The molecular weight of the mAb (MW_{mAb}) is assumed to be 150 kDa.
The N-glycosylation process progresses in Golgi, where the reactions catalyzed by ten enzymes in four compartments. Mass balance for each individual glycan in a compartment (except from $[G_{1,1}]$) is described by the following equation

$$\frac{d\,[G_{i,j}]}{dt} = \sum_{i=1}^{\#reactions} S_G \cdot r_{G_{i,j}} + k_{t_j} \cdot (G_{i,j-1} - G_{i,j}) \tag{A.19}$$

where the concentration of glycan structure i in the compartment j is $G_{i,j}$. The first term in the right-hand side shows the Golgi glycosylation reaction kinetics, and S_G is the stoichiometric coefficient matrix. The term k_{t_j} indicates the transport rate to or from a compartment. For the single nucleotide substrate enzymes like ManI and ManII the Michaelis-Menten kinetics with glycan substrate competition are considered [24]

$$r_{G_{i,j}} = \frac{r^{max}_{G_{e,i}} \cdot [G_{i,j}]}{K^G_{e,i} \cdot \left(1 + \sum_{l=1,l\neq i}^{n} \frac{[G_{l,j}]}{K^G_{e,l}}\right)} \tag{A.20}$$

For multiple substrate enzymes (i.e., GnT I-III, FucT, GalT, SiaT) the competition terms for both glycans and nucleotides is considered in the kinetic rate [24]

$$r_{G_{i,j}} = \frac{r^{max}_{G_{e,i}} \cdot [G_{i,j}] \cdot [NS_i]}{K^G_{e,i} \cdot K^S_{e,i} \cdot \left(1 + \sum_{l=1,l\neq i}^{n} \frac{[G_{l,j}]}{K^G_{e,l}} + \frac{[NS_{l,j}]}{K^S_{e,l}} + \sum_{l=1,l\neq i}^{n} \frac{[G_{l,j}]}{K^G_{e,l}} \cdot \frac{[NS_{l,j}]}{K^S_{e,l}}\right)} \tag{A.21}$$

where, $r^{max}_{G_{e,i}}$ is maximum specific rate coefficient and $K^G_{e,i}$ and $K^S_{e,i}$ are dissociation constants for glycan and donor co-substrate, and n is the number of reactions catalyzed by the same enzyme.

The calculated concentration of each glycan leaving Golgi is then multiplied by the viable cell density to give the accumulated glycan concentration in the culture supernatant.

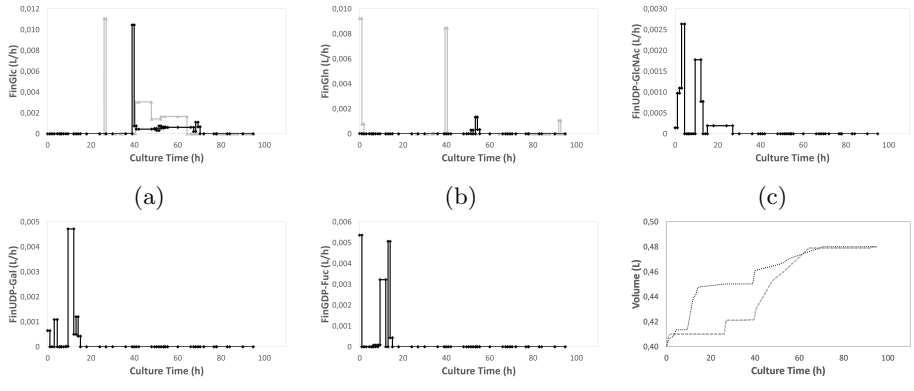
B Supplementary optimization results

Table B1: Optimization convergence details as reported by gProms [1]. Values with * indicate termination following lack of improvement in optimization variables and objective function. No improvement tolerance is set to 1E-12 (default) All CPU times are in [s].

	Optimization Status	**CPU Time**
CS1a	optimal	186.1
CS1b	optimal*	9255.0
CS2a	optimal*	993.4
CS2b	optimal*	145651.0
CS3a	optimal	24.3
CS3b	optimal	323.1

Table B2: Concentration of nutrients in the feed. Values with * indicate an active bound. All concentrations are measured in [mM].

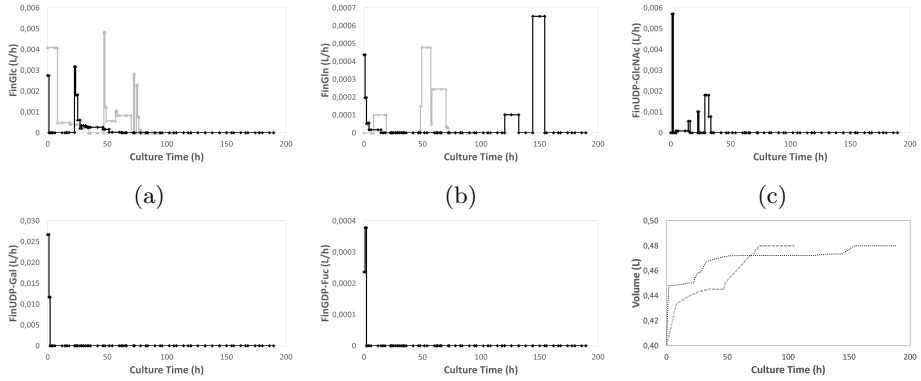
	$\mathbf{c_{inGlc}}$	$\mathbf{c_{inGln}}$	$\mathbf{c_{inUDPGlcNac}}$	$\mathbf{c_{inUDPGal}}$	$\mathbf{c_{inGDPFuc}}$
CS1a	160.3	20.0	N/A	N/A	N/A
CS1b	400.0*	200.0*	29.2	296.4	0.7
CS2a	277.0	199.4	N/A	N/A	N/A
CS2b	400.0*	200.0*	100.0*	95.7	0.0*
CS3a	59.1	112.1	N/A	N/A	N/A
CS3b	400.0*	200.0*	1.6	35.8	0.0*



(a) (b) (c)

(d) (e) (f)

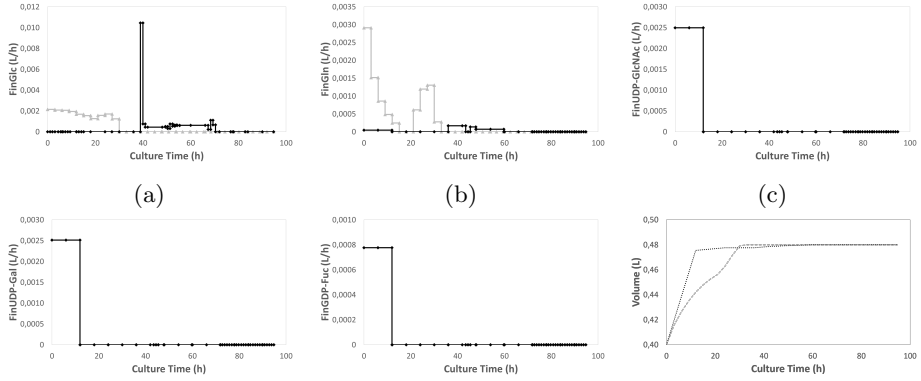
Fig. B.1:
CS1 - Feeding profiles and volume variation for optimized without QA (light grey) and optimized with QA (black) cases.



(a) (b) (c)

(d) (e) (f)

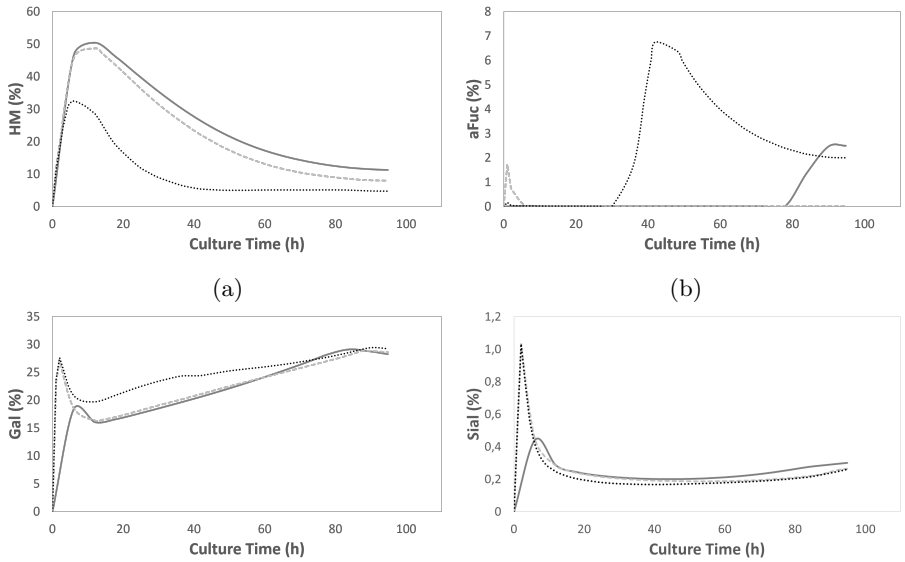
Fig. B.2:
CS2 - Feeding profiles and volume variation for optimized without QAs (light grey) and optimized with QAs (black) cases.



(a) (b) (c)

(d) (e) (f)

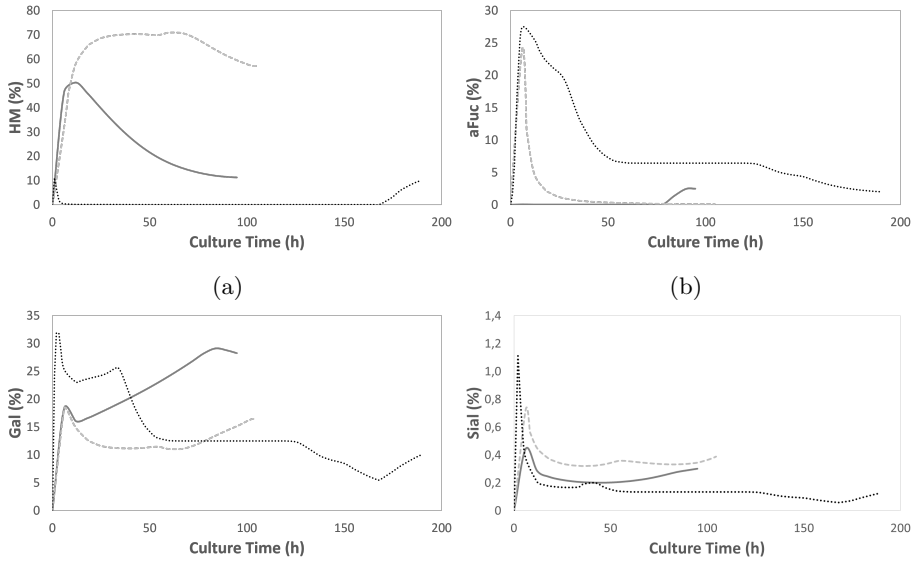
Fig. B.3:
CS3 - Feeding profiles and volume variation for optimized without QAs (light grey) and optimized with QAs (black) cases.



(a) (b)

(c) (d)

Fig. B.4:
CS1 - Glycan distribution profiles for unoptimized (solid, dark grey) optimized without QAs (dashed, light grey) and optimized with QAs (dotted, black) cases.



(a) (b)

(c) (d)

Fig. B.5:
CS2 - Glycan distribution profiles for unoptimized (solid, dark grey) optimized without QAs (dashed, light grey) and optimized with QAs (dotted, black) cases.

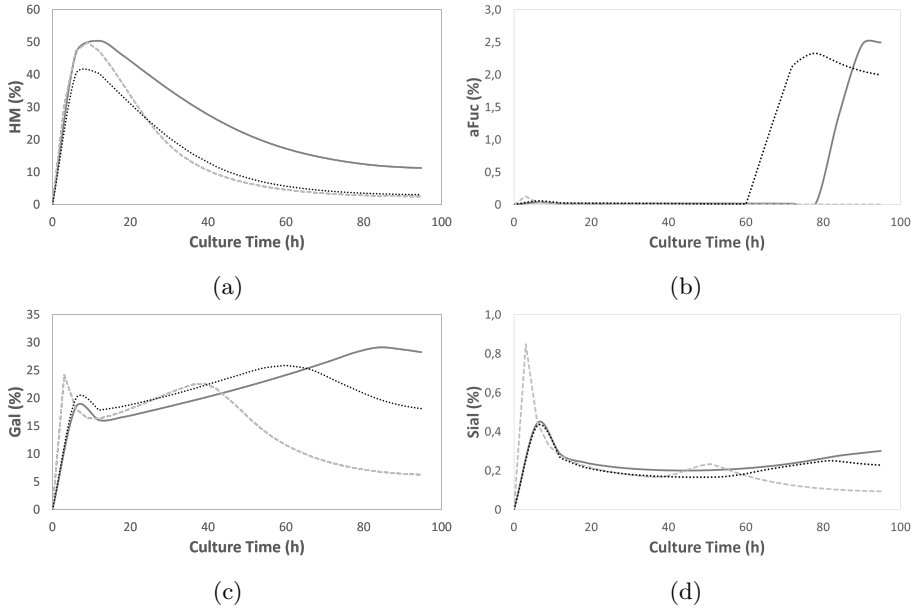


Fig. B.6:
CS3 - Glycan profiles for unoptimized (solid, dark grey) optimized without QAs (dashed, light grey) and optimized with QAs (dotted, black) cases.